DNA fingerprinting and genetic relationships among willow (*Salix* spp.)

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By

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ABSTRACT

Given that morphological identification of willow is difficult, willow lines being investigated for their suitability for use as short rotation crops for biomass production in Saskatchewan were investigated with various molecular techniques as possible tools for DNA fingerprinting. Flow cytometry was used to assess variation in nuclear DNA content and thus ploidy level of the lines of the five species (*Salix purpurea*, *Salix eriocephala*, *Salix sachalinensis*, and *Salix dasyclados*) and three hybrids (*S. purpurea x S. miyabeana, S. sachalinensis x S. miyabeana, S. viminalis x S. miyabeana*). The DNA content varied between 1.14 and 3.00pg. Ploidy levels of the species varied from triploid to hexaploid while all hybrids were tetraploid. RAPD and ISSR marker systems were used to assess genetic and taxonomic relationships among all willow lines. Of 90 RAPD primers tested, 60 were selected and 99 polymorphic bands scored. Of 35 ISSR primers tested, 19 were selected and 35 polymorphic bands scored. Both RAPD and ISSR dendrograms clustered together lines belonging to the same species and same hybrid combination. A combination of strong and reproducible RAPD and ISSR bands was used to develop identification keys for lines belonging to the same species.

The ribosomal RNA gene region, including the entire 5.8S RNA gene and the internal transcribed spacers (ITS1 and ITS2) was amplified and sequenced to assess sequence homology between the five species. The total length of the amplified region was 601bp, with the ITS1, 5.8 S and ITS2 being 223, 163, and 215bp respectively. Intra- and inter-species SNPs were observed, 6 within ITS1, and 3 within ITS2. No polymorphisms were found in the 5.8S gene. The low rate of variation within the sequenced ITS fragment between species supports the monophyly of the five species involved in this study, and confirms their belonging to the subgenus *Caprisalix*. SCAR primers were designed from species-specific polymorphic nucleotides and applied to the willow collection to test their use for species identification. A species identification key based on SNPs is proposed.

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LIST OF ABREVIATIONS

AFLP	amplified fragment length polymorphism
bp	base pair
CTAB	cetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylene-diamine-tetraacetic acid
IEA	international energy agency
IGS	intergenic spacer
IPCC	intergovernmental panel on climate change
ISSR	inter simple sequence repeat
ITS	internal transcribed spacer
NTS	non-transcribed spacer
NTSYS	numeric taxonomic and multivariate analysis systems
OTU	operational taxonomic unit
PCR	polymerase chain reaction
pg	picogram
RAPD	random amplified polymorphic DNA
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SAHN	sequential, agglomerative, hierarchical, and nested clustering method

SCAR	sequence characterised amplified regions
SNP	single nucleotide polymorphism
SRW	short rotation willow
SSR	simple sequence repeats
TBE	tris base, boric acid and EDTA
UPGMA	unweighted pair group method with arithmetic mean
UV	ultraviolet

1-INTRODUCTION

A number of willow lines are being investigated for their suitability for use in Saskatchewan as short rotation crops for biomass production, and to assess the impact on the biogeochemical cycle of carbon and nitrogen of converting agricultural landscapes back to woody vegetation. The material involved in that study includes five lines of *Salix purpurea,* three lines of *Salix miyabeana,* four lines of *Salix eriocephala,* one line of *Salix dasyclados,* one line of *Salix sachalinensis,* inter-species hybrids (four *S. purpurea x S. miyabeana,* four *S. sachalinensis x S. miyabeana,* eight *S. viminalis x S. miyabeana*), and five lines of unknown origin.

Morphological identification of willow lines and species is often difficult due to intra-specific variation, superficial similarities and developmental variability. These difficulties cause concerns about certifying that the correct species or line is being used in the program. It will be important to ensure that the correct lines or species are used when preparing propagating material for plantations. This work aims to use molecular marker techniques to assess genetic relationships among those willow lines, and to identify molecular marker banding patterns (DNA fingerprints) that can accurately differentiate individual lines and species.

2-LITERATURE REVIEW

2-1 Climate change

For more than one century, modern societies have been deeply dependent on fossil carbon for fuel production and for chemicals ranging from plastic polymers to drugs and food additives (Benning and Pichersky, 2008). Oil production has increased from less than 1 million tonnes a year in 1870, to more than 3 billion tonnes a year (Bennewitz, 2009). These industrial activities have caused important releases of harmful organic and inorganic compounds to the environment, including greenhouse-effect gases, heavy metals, and petroleum hydrocarbons (Wilcke and Amelung, 2000; Huang et al., 2005; Rodríguez et al., 2008;). Since the Industrial Revolution, atmospheric carbon dioxide concentration has risen from 280 to 365 ppm, and predictions are that it could reach 700 ppm by the second half of the 21st century (Houghton et al., 1996; Lamlom and Savidge, 2003). The CO_2 layer helps to retain heat that would otherwise be lost to space. An increase of atmospheric carbon dioxide concentration consequently leads to global warming (Bennewitz, 2009).

In addition, forest ecosystems are continuously destroyed releasing CO_2. Between 1990 and 1997, 5.8 ± 1.4 million hectares of the world's humid tropical forest were lost each year, with a further 2.3 ± 0.7 million hectares of forest visibly degraded (Achard et al., 2002). In Canada, the main causes of deforestation are the conversion of forest to agricultural and urban lands. The deforestation rate in Canada is estimated at 546-805 km^2 per year linked with a yearly release of 9.5-14.0 Mt carbon dioxide (Robinson et al., 1999). The combined effect of forest loss and intensive use of fossil fuel leads to an

increase of atmospheric carbon dioxide concentration and global warming (Benning and Pichersky, 2008; IEA, 2002), which is the main threat to human development and the future of the planet (Houghton et al., 1996). The Intergovernmental Panel on Climate Change (IPCC) predicts an anthropogenic warming increase between 1.4 and 5.8°C over the 21st century (IPCC, 2001). In Canada, the annual mean temperature increased by 1.2°C between 1955 and 2005 (Vincent et al., 2007).

Many countries including Canada have ratified international agreements such as the Kyoto protocol to reduce greenhouse gas emissions and mitigate human impact on global warming. Those agreements rely on the identification of renewable sources of energy that can substitute for the use of fossil fuel energy (reducing fossil fuel carbon dioxide emissions to the atmosphere), as well as the adoption of sustainable environmental management practices (Smart et al., 2005; IEA, 2002).

2-2 Biomass, a valuable source of renewable energy

Concerns about global warming, air and water pollution associated with the use of fossil fuels, have brought growing interests in the development of bio-energy and bio-products in western countries (Keoleian and Volk, 2005). Global energy-use projections predict that biomass has the potential to become one of the major primary energy sources in the coming decades and modernized bio-energy systems are suggested to be important contributors to future sustainable energy systems in both industrialized and developing countries (IEA, 2002; Berndes et al., 2003).

Biomass energy is unique among renewable energy sources (wind, water and solar energy) in that it can be both a source of electricity, and a source of liquid and

gaseous fuels (Graham et al., 1992). Renewable biomass feedstock can come from various sources including forest, agricultural crops and short rotation woody crops (Abrahamson et al., 1998; Volk et al., 2004). Short rotation woody crops show multiple environmental and rural development benefits, and are promoted as a potential source of significant amounts of renewable biomass that could offer landowners an attractive alternative use for marginal croplands (Berndes et al., 2003).

2-2-1 Energy production from biomass

Biomass represents solar energy stored in organic compounds (Hautala et al., 1979). Through the process of photosynthesis, green plants use chlorophyll to absorb and convert solar energy into the chemical energy used to reduce atmospheric CO_2 into carbohydrates (sugars, starch, cellulose, and lignin). The degradation of carbohydrates releases energy, minerals and the same amount of CO_2, and H_2O used during their synthesis (Figure 2.1).

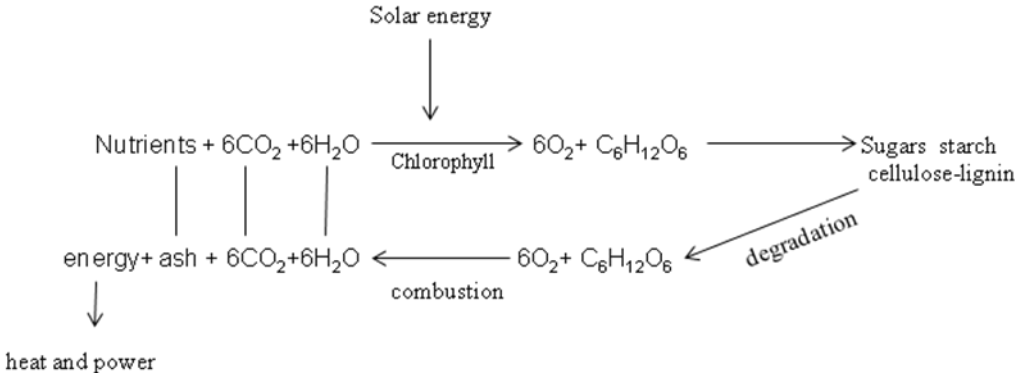


Figure 2.1: Cycle of biomass energy (Miyamoto, 2009)

Many technologies can be used to generate energy and power from woody biomass. Direct combustion technologies can be used to convert biomass fuels into various forms of energy (mechanical energy, heat or electricity), using processes similar to that employed for fossil fuels (Dumbleton, 1997). The process involves the burning of biomass in a furnace or a boiler to generate heat and steam. Steam can then run through a turbine to produce mechanical energy. If the turbine is connected to an electrical generator it produces electricity.

Gasification technology uses high temperature and high pressure for partial oxidation of biomass into combustible gas (Nordin and Kjellström, 1996). The gas fuel resulting from this process consists mainly of carbon monoxide and hydrogen with small amounts of methane, ethane and ethylene (Dumbleton, 1997).

Pyrolysis is the process where biomass is combusted at high temperatures and decomposed in the absence of oxygen. It results in the production of solid, liquid and gaseous products. Pyrolysis oil can be combusted in engines to produce heat and power (Bidini et al., 2003).

Research is also underway to develop bacterial and yeast strains that can be used for bio-ethanol production from lignocellulosic sugars (Zaldivar et al., 2001).

2-2-2 Willow, a dedicated crop for biomass and renewable energy production

Woody biomass crop development in northern temperate areas has focused on willow shrubs and poplar (Abrahamson et al., 1998; Perttu, 1999; Keoleian and Volk, 2005). Willow, however, exhibits more diversity (number of species) and grows in a wider range of environments compared to poplar (Verwijst, 2001).

Most willows are characterised by particular physiological assets that make them suitable for biomass and energy production (Kuzovkina and Quigley, 2005). Willows exhibit high growth rates and biomass productivity (Keoleian and Volk, 2005). The growth rate of willow is superior to most cool temperate tree species, with an annual growth of 1 to 4 m during the first five years after planting, depending on soil fertility and moisture regimes (Wilkinson, 1999). Willow is considered among the most promising biomass fuels in many temperate regions. In Quebec, Labrecque and Teodorescu, (2003) reported 71.3 tonnes of dry matter yield per hectare three years after planting. Willow plantations are clonally propagated and can be harvested every three to five years, after which the cut stools regenerate 5-14 new stems to provide another harvest, and may continue to do so up to eight times, giving a productive lifespan of 20-32 years (Volk et al., 2004). This provides farmers with a new crop that produces a regular income and faster returns than those associated with conventional forestry (Volk et al., 2004).

Energy produced from short rotation willow (SRW) biomass is renewable, because the growth of new plants and trees replenishes the supply. Carbon dioxide emission from burning of SRW biomass is neutral, because it is recycled via photosynthesis in stands growing to replace those harvested (IEA, 2002). Carbon sequestration in the soil increases in SRW plantations, and net emissions of carbon dioxide may even be negative (Grogan and Matthew, 2002).

2-2-3 Other uses of willow

Willow plantations can be used as a biological filter for wastewater treatment via a system based on soil filtration of effluents, the degradation of organic particles by micro-organisms, and the uptake of nutrients and heavy metals by willow trees (Pulford and Watson, 2003). The bio-filtration system offers many advantages including recycling of nutrients, a less expensive purification system for water companies, and higher profitability for willow growers due to a lower cost for fertilisers and a higher yield. The use of such willow as wood fuel would allow heavy metal recovery through the scrubbing of smoke gases and proper handling of ashes (Pulford and Watson, 2003).

Willows possess the major requirements for plant survival in degraded ecosystems including the ease of vegetative propagation, the ability to re-establish from cut stumps and produce a dense coppice (Volk et al., 2004), and the ability to accumulate high levels of toxic metals (Perttu, 1999; Mirck et al., 2005). Willows are recommended for remediation of oil-mining areas because of their potential to rapidly sequester pollutants and re-establish green cover (Kuzovkina and Quigley, 2005). The colonization of environmentally disturbed sites by willow species supports the establishment of pioneer species, fasters the recovery of damaged ecosystems and the re-establishment of natural ecological complexity (Kuzovkina and Quigley, 2005). Many invertebrates, birds and animals feed on willow and support a large food chain for higher trophic level organisms (Kennedy and Southwood, 1984). Willow energy plantations can create new habitat opportunities for wildlife in degraded ecosystems (Kuzovkina and Quigley, 2005).

2-3 The genus *Salix*

2-3-1 Origin and distribution

Plants belonging to the genus *Salix* are among the earliest recorded pre-ice age flowering plants (Newsholme, 2002). The genus *Salix* originally arose in the subtropics, and then advanced slightly into the tropics, expanded into the temperate regions, and later into the Artic. *Salix* species are now mostly distributed in the northern temperate zones (Europe, Asia, North America). Species diversity is richest in China (270 species) and Russia (120 species). There are about 65 species in Europe and 160 species in North America (Kuzovkina and Quigley, 2005). Only 3 species are native to central and South America (Argus, 1986). In tropical Africa, there are about 12 species most of which are endemic only to Africa and are extremely local (Newsholme, 2002).

2-3-2 Description and current classification

The genus *Salix* belongs to the family Salicaceae. Members of this family are trees and shrubs (Watson and Dallwitz, 1992). Flowers are aggregated in inflorescences called catkins which are usually pendulous or erect (Dorn, 1976). Originally, Linnaeus (1763) described and divided this family into two genera, the genus *Populus* and the genus *Salix*. Nakai (1920) described a new genus *Chosenia,* which bears intermediate characters between these genera (Table 2.1).

Table 2.1 Morphological characters for genus identification in the family Salicaceae (Watson and Dallwitz, 1992)

	Salix	***Chosenia***	***Populus***
Growth	Sympodial	Sympodial	Monopodial
Bud scale	1	1	Several
Catkin	♀ erect	♀ erect rarely pendulous	♂ pendulous
	♂ erect	♂ pendulous	♀ pendulous

Salix species are dioecious trees and shrubs, with individuals bearing either all male or all female flowers arranged in catkins (Dorn, 1976). Plants show traits of insect pollination including stiff erect catkins and the availability of nectar, but also traits of wind pollination, including the small size of flowers, the absence of perianth and the early flowering in the spring, before leaf unfolding (Karrenberg et al., 2002). The genus *Salix* is very heterogeneous, comprising 400 to 500 species with considerable variation in size and growth forms (Stott, 1984). The most widely accepted classification is that of Skvortsov (1968). He recognised three main subgenera:

Subgenus *Salix:* (true willow), members of this subgenus are the most primitive of the genus *Salix* (Dorn, 1976). They are upright pendulous or semi-pendulous trees and large shrubs with narrow serrate leaves. Catkins are made up of uniformly coloured flowers. Male flowers have two or more nectaries, while female flowers have one or two. This subgenus is subdivided into eleven sections (Newsholme, 2002).

Subgenus *Caprisalix* (Dumort.) Nasarov: also known as osiers and sallow, they are shrubs and small trees with great variation in leaf forms. Catkins are sessile or sub-sessile, flowers are precocious; male flowers have two distinct stamens and female flowers have only one nectary. This subgenus is subdivided into fourteen sections

(Newsholme, 2002). Most willows used in crop systems belong to this subgenus. More than 125 species are known worldwide (Volk et al., 2004).

***Chamaetia* (Dumort.) Nasarov:** Plants are dwarf or procumbent shrubs of less than 1m high, leaves are less than 10 cm long, Catkins are borne by leafy branchlets, and male flowers have two stamens, occasionally one. This subgenus is subdivided into seven sections (Newsholme, 2002).

The taxonomy of the genus *Salix* has been continuously under revision (Skvortsov, 1968; Meikle, 1984). Many factors contribute to difficulties in the identification of *Salix* species, including high morphological variability, widespread hybridization and introgression, and variation in ploidy levels (Argus, 1997). Within the genus *Salix,* quantitative characters like length of catkins, absence or presence of flowering branchlets, and ovary length vary with developmental stage. Some structures like stipules or floral bracts may be lost with maturity (Argus, 1986). In addition, the use of floral characteristics for identification is limited because of the extremely reduced size of the flowers (Argus, 1997).

Hybridisation is an important source of variability within the genus *Salix* (Dorn, 1976). Inter-specific crosses frequently occur in the wild and in cultivation. Hybrids may be imperfectly intermediate or highly variable, resulting in an interpretation that unrecognized hybrid plants are part of the morphological variation. Most hybrids are fertile and can cross with other species and hybrids, bringing more problems associated with classification and identification (Stott, 1984).

2-3-3 Ploidy variation in *Salix* species

Salix species are dioecious. Pollination is either entomophilous or anemophilous. The basic chromosome number is 19. The ploidy level varies both between and within members of the same species (Håkansson, 1955; Darlington and Wylie, 1961). The chromosome number ranges from (2n=38) for diploids; (2n=57) for tripoids; (2n=76) for tetraploids; (2n=95) for pentaploids; (2n=114) for hexaploids; to (2n=152) for octoploids (Suda and Argus, 1968). Over 40% of *Salix* species are polyploids. Polyploidization may have occurred independently several times in *Salix* (Dorn, 1976). The evaluation of chromosome number and ploidy level can help to recognise the parentage of hybrids involving species with different ploidy levels, and to delimit taxa unrecognised morphologically (Suda and Argus, 1968; Chmelar, 1979).

2-3-4 Molecular genetics of the genus *Salix*

Research on the molecular genetics of *Salix* species is recent. Barker et al., (1999) used random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analysis to characterise the genetic diversity in potential biomass of willows. They reported that RAPD analysis can be performed with relatively crude DNA, but differences in DNA purity between samples can be a cause of un-reproducible profiles. Twenty RAPD primers were able to distinguish up to 10 of the 20 lines involved in that study. The AFLP technique revealed more genetic diversity and was better able to discriminate closely related lines. High quality DNA was essential for complete digestion with the restriction enzymes required for AFLP. Hanley et al., (2002) used 291 AFLP and 39 microsatellite markers to develop the genetic linkage map of *Salix viminalis.* Barker et

al., (2003) developed microsatellites from an enriched library of *Salix burjatica*, most of which cross-amplified diverse *Salix* species. Sulima et al., (2009) used RAPD to reveal the genetic diversity and fingerprint lines of *Salix purpurea.* Nucleotide sequences of ribosomal RNA gene regions (ITS1, 5.8S and ITS2) were used by Leskinen and Alstrom, (1999) to study the molecular phylogeny of *Salicaceae* and closely related *Flacourtiaceae.* They reported a low inter-specific variation in ITS sequence among members of the family *Salicaceae*.

2-4 Genetic markers and fingerprinting

A genetic marker can be defined as a measurable character that can detect variation in either the phenotype or the genotype (King and Stansfield, 1990). Genetic markers can be based on visually assessable traits (morphological markers), on gene products (biochemical markers), or rely on a DNA assay (DNA markers).

Morphological markers were very useful to early geneticists for the study of classical heredity. However, their number is limited, and their expression can vary over a range of environments or be influenced by pleiotropic interactions and developmental stage (Staub et al., 1996).

Molecular markers are DNA sequences that reveal sites of variation in DNA. DNA sequence variation generally arises from point mutations (substitutions) rearrangements, insertions, deletions, and errors in replication (Paterson, 1996). They provide a new supply of character differences that can be detected in laboratory assays. There are two categories of molecular markers: Biochemical markers based on the detection of differences in proteins through gel electrophoresis, and DNA markers based

on detection of variation in DNA (Winter and Kahl, 1995). Compared to morphological and biochemical markers, DNA markers are more abundant, more polymorphic, reproducible, discriminating and not subject to environmental changes; they can be detected in any tissue of the organism at any developmental stage (Winter and Kahl, 1995).

2-4-1 DNA markers and fingerprinting

Many different DNA marker systems are now widely used. They are either hybridization or polymerase chain reaction (PCR) based (Collard et al., 2005). The most commonly known hybridization based DNA marker system is restriction fragment length polymorphism (RFLP). This system is based on digestion of genomic DNA with restriction enzymes. The resulting fragments are separated by gel electrophoresis, blotted onto a filter, and probed with a small fragment of radio-labelled cloned genomic or complementary DNA. Through autoradiography, probes can be visualized on the filter and banding patterns used for genetic analysis (Beckmann and Soller, 1986). Genetic variations come from gain or loss of restriction sites between genomes as a result of mutations, insertions, inversions, or deletions (Doebley and Wendel, 1989). However, RFLP marker systems have some limitations. The process is laborious, time consuming and requires a relatively large amount of good quality DNA (Beckmann and Soller, 1986).

The invention of PCR in 1984 by Mulli, (1990) brought the development of new marker technologies that were able to surmount some of the limitations of RFLP (Tautz and Renz, 1984; Williams et al., 1990). The random amplified polymorphic DNA

technique (RAPD) is a PCR-based technique in which a single short oligonucleotide (10 bp) of arbitrary sequence anneals to the genomic DNA at different sites on complementary strands of the DNA template. If the priming sites are within an amplifiable range of each other, a discrete DNA product is formed through thermo-cyclic amplification (Williams et al., 1990). Amplified fragments (within the 0.5-5 kb range) can be separated by gel electrophoresis and visualised by UV light after staining with ethidium bromide. The polymorphisms are identified as the presence or absence of a given amplification product in the gel, and result from variation in the sequence of the primer binding sites, or from chromosomal rearrangements within the amplified sequence, altering the size or the successful amplification of the target site (Williams et al., 1990). No prior sequence information is required for the design of RAPD primers and they can easily be applied to any organism (Hadrys et al., 1992). The RAPD technique has a wide range of applications, including genetic similarity analysis (Sulima et al., 2009), fingerprinting (Castiglione et al., 1993), and genetic mapping (Hadrys et al., 1992).

Microsatellites or simple sequence repeats (SSRs) are loci where di- tri- or tetra-nucleotide sequences of DNA are repeated in tandem arrays (Powell et al., 1996). SSRs are scattered throughout genomes and exhibit a higher mutation rate compared to other regions of DNA, due to slipped strand mispairing (slippage) during DNA replication on a single DNA strand (Jeffreys et al., 1985). The number of times the sequence is repeated at a particular locus often varies between individuals, populations, and between species (Jeffreys et al., 1985). The development of microsatellite markers involves several steps including the development of a genomic DNA library, the identification of microsatellite

loci and the design of suitable forward and reverse primers for amplification (Semagn et al., 2006).

Inter simple sequence repeats (ISSRs) are arbitrary multilocus markers produced by PCR amplification of genomic DNA with single microsatellite based primers (Tautz and Renz, 1984). The ISSR technique is a RAPD-like approach that accesses variation in the numerous microsatellite regions dispersed throughout genomes (Joshi et al., 2000). The primer locates two microsatellite regions within an amplifiable distance on the DNA template strands, and the PCR reaction generates a band of a particular size representing the stretch of DNA between the microsatellites (Semagn et al., 2006). ISSRs are highly polymorphic, do not require prior genomic information for primer design, and show a higher reproducibility than RAPD and RFLP approaches (Tsumura et al., 1996). ISSR markers have been used to assess genetic diversity and to fingerprint many crops (maize, beans, barley) (Kantety et al., 1995; Metals et al., 2000).

Amplified Fragment Length Polymorphism (AFLP) involves the digestion of genomic DNA followed by ligation of adaptors to the ends of digested fragments, and the selective PCR amplification of a set of fragments (Hanley et al., 2002). Amplified fragments are separated on acrylamide gels and visualized by autoradiography, silver staining or fluorescent sequencing equipment (Vos et al., 1995). AFLP is a multilocus DNA profiling technique in which it is possible to obtain information for many different loci in a single assay. AFLP produces a large amount of data from a single experiment, and is a useful method for distinguishing between closely related individuals (Vos and Kuiper, 1997). However, AFLP requires high quality DNA for complete digestion.

Single nucleotide polymorphisms (SNPs) are DNA sequence variations occurring when a single nucleotide in the genome differs between members of a species. They can occur in both coding and non-coding regions, and represent the largest set of sequence variants in most organisms (Kwok et al., 1996). Specific oligonucleotide primers can be designed from SNPs to develop sequence characterized amplified region (SCAR) markers through PCR amplification of specific DNA fragments (McDermott et al., 1994). The SNP marker approach requires prior sequence information and is increasingly used as DNA sequences become accessible in databases (Kurt et al., 2005).

2-4-2 Ribosomal RNA genes in phylogenetic analysis and fingerprinting

Ribosomal RNA genes **(**rDNA) are DNA sequences responsible for the synthesis of ribosomal RNA (rRNA) (Shiue et al., 2009). These genes are tandemly repeated along chromosomes and exist in thousands of copies. Coding regions (18S, 5.8S, 5S, and 28S) are highly conserved between families, genera and species. Non-coding regions, (the non transcribed spacer (NTS), the intergenic spacer (IGS), and the internal transcribed spacer (ITS)) are much more variable, but undergo rapid concerted evolution known as molecular drive, that promotes intra-genomic uniformity (Leskinen and Alstrom, 1999). The characterisation of spacers can be used for inter-species comparison, genetic variations and phylogenetic relationships among taxa (Scoles et al., 1987; Shiue et al., 2009).

2-4-2-1 Structural organization of ribosomal RNA genes

There are two classes of rRNA gene repeats, the 5S repeats and the 18S-5.8S-28S repeats (Flavell, 1986). The 18S-5.8S-28S repeats are clustered in the nucleolar organizing regions of satellited chromosomes. Consecutive repeats are separated by the intergenic spacer (Hizume et al., 1992). Hotta and Miksche, (1974) reported between 10,000 to 30,000 copies of the 18S-5.8S-28S repeats in *Pinus* species. The internal transcribed spacer 1 (ITS1) is located between the18S and 5.8S genes and the ITS2 between 5.8S and 28S genes (Figure 2.2).

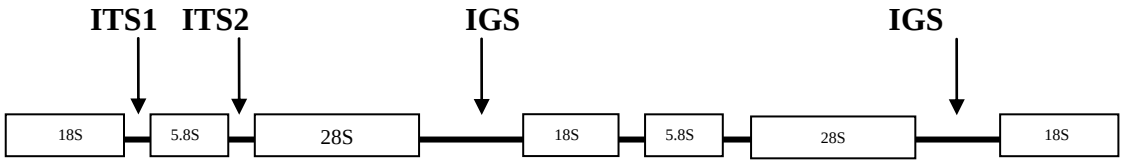


Figure 2.2: Structure of the 18S-5.8S-28S rRNA gene repeats

In most angiosperms studied, the 5S rRNA gene units are located at multiple chromosomal sites (Scoles et al., 1987). Consecutive 5S units are separated by 100-380 bp long spacers (NTS) (Figure 2.3) (Scoles et al., 1987).

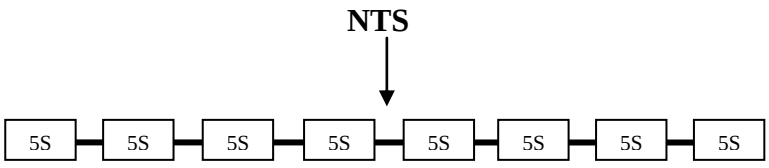


Figure 2.3: Structure of the 5S rRNA gene repeats

2-5 Measuring ploidy level

Salix chromosomes are small and are difficult to count accurately (Suda and Argus, 1968; Thibault, 1998). Measurement of nuclear DNA content has been used as rapid and accurate estimation of *Salix* chromosome number and ploidy levels (Thibault, 1998). Flow cytometry involves preparation of aqueous suspensions of intact nuclei whose DNA is stained using propidium iodide (Arumuganathan and Earle, 1991). Fluorescence intensities of the stained nuclei are measured with a flow cytometer and values for nuclear DNA content estimated by comparing fluorescence intensities to those of appropriate standards (Doležel and Bartos, 2005). Flow cytometry has become widely used for the measurement of the nuclear DNA content and ploidy screening in plants, and has proved to be rapid and more efficient than the previous methods including Feulgen microspectrophotometry and DNA image densitometry (Doležel and Bartos, 2005).

2-6 Methods of construction of dendrograms

A dendrogram is a tree-like diagram frequently used to illustrate the arrangement of the clusters produced by hierarchical clustering (Hillis et al., 1996). It helps to visualise the basic representation of the overall similarity or evolutionary history relationships among operational taxonomic units (OTU) (individuals, cultivars, species or populations) (Hillis et al., 1996). Each step of the tree represents a fusion of two branches into a single one, and the branches represent clusters obtained on each step of hierarchical clustering (Hillis et al., 1996). Three main approaches used to develop a dendrogram are the parsimony analysis approach, the maximum likelihood approach and the distance matrix approach (Felsenstein, 1988; Crawford, 1990). In the distance matrix approach,

the tree is generated with an algorithm based on functional relationships among distance values. Several methods are based on this approach, including the Wagner distance method, the least square method, the neighbour joining method and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The UPGMA is known as the simplest and the most frequently used method, especially for allelic frequency data (Crawford, 1990). It is based on a sequential algorithm that generates the tree in 2 steps. First, two most similar OTU are identified and clustered in a single composite OTU, which will then be grouped with the next most similar one.

3-THE USE OF FLOW CYTOMETRY, RAPD AND ISSR MARKERS FOR FINGERPRINTING AND GENETIC DIVERSITY ANALYSIS OF *Salix* SPECIES

3-1 Abstract

Flow cytometry was used to assess variation in nuclear DNA content and ploidy level of 35 willow lines. The DNA content varied between 1.14 and 3.00pg. There was slight variation among lines of the same species. Ploidy levels of the species varied from triploid to hexaploid. All hybrid lines came from crosses between tetraploid parents.

RAPD and ISSR marker systems were used to assess genetic and taxonomic relationships among the willow lines. Of 90 RAPD primers tested, 60 were selected and 99 polymorphic bands were used for genetic relationships analysis. Thirty five ISSR primers were tested, 19 produced polymorphic bands and a total of 35 polymorphic bands were used for genetic relationships analysis. Both RAPD and ISSR dendrograms clustered together lines belonging to the same species. Hybrid lines were grouped together. Both dendrograms clustered the line Juliet with lines of *S. eriocephala*, Hotel with lines of *S. purpurea* and India with lines of *S. dasyclados*. The 2C nuclear DNA values of Juliet and Hotel also fell within the range of 2C nuclear DNA values of *S. eriocephala* and *S. purpurea* lines respectively. A combination of strong and reproducible RAPD and ISSR bands were used to develop identification keys for lines belonging to the same species. These results show that RAPD, ISSR marker techniques and flow cytometry can be efficiently used to discriminate among closely related willow lines.

3-2 Introduction

Biomass willow lines are being investigated for their suitability as short rotation biomass energy crops in Saskatchewan. Willow lines being used in that investigation can be grouped into five species (*Salix purpurea*, *Salix miyabeana*, *Salix eriocephala*, *Salix dasyclados*, *Salix sachalinensis*) and inter-species hybrids (*S. purpurea x S. miyabeana*, *S. sachalinensis x S. miyabeana, S. viminalis x S. miyabeana*) (Table 3.1), all belonging to the subgenus *Caprisalix*. Observation of this material in the field showed that different lines of the same species tend to be very similar morphologically. Inter-specific hybrids bear intermediate characters between parents, but exhibit morphological variability. Some hybrids can easily be misidentified as parents. Because of their unknown origin, five lines included in this investigation could not be accurately classified.

The genus *Salix* is known to be one of the few woody genera with a large number of polyploid taxa (Suda and Argus, 1968; Chmelar, 1979). The evaluation of ploidy level can therefore be useful to delimit taxa and to recognise the parentage of hybrid crosses involving species with different ploidy levels (Chmelar, 1979). Ploidy levels can be deduced from nuclear DNA values measured with flow cytometry (Morgan et al., 1995; Thibault, 1998).

DNA markers reveal sites of variation at the DNA level, and can be useful in discriminating closely related species and breeding lines (Raina et al., 2001; Patzak, 2001; Eckstein et al., 2002). Compared to morphological markers, DNA markers are more abundant, more polymorphic, reproducible, and not subject to environmental changes (Winter and Kahl, 1995). The advent of PCR favoured the development of molecular techniques such as RAPD (William et al., 1990), and ISSR (Tautz and Renz,

1984). Both techniques require small amounts of DNA, and a single primer to amplify numerous discrete loci in the genome (Tingey et al., 1993; Tautz and Renz, 1984). RAPD and ISSR protocols are less time and labour consuming than RFLP and AFLP. No sequence information on amplified regions is required. RAPD and ISSR primers drive the amplification of numerous loci in the genome and represent efficient tools to screen for genetic polymorphisms (Heun et al., 1994; Tautz and Renz, 1984).

The objective of this study was: i) to estimate DNA content and ploidy level of individual lines using flow cytometry, ii) to utilize RAPD and ISSR marker techniques to generate polymorphic banding patterns for genetic relationsip analysis among the lines and to utilize this information to develop DNA markers for the lines.

3-3 Material and methods

3-3-1 Plant material

Thirty-five willow lines from the biomass willow collection grown by the Center for Northern Agroforestery and Afforestation at the University of Saskatchewan were used in this investigation (Table 3.1).

Table 3.1 List of biomass willow lines utilized, their ID and species designation

Species	lines ID / names	Codes
Salix purpurea	• 94001	17
	• Fish Creek (Clone ID 9882-34)	18
	• 9882-41	19
	• Onondaga (Clone ID 99113-012)	20
	• Allegany (Clone ID 99239-015)	21
Salix eriocephala	• 9837-77	23
	• 00X-032-094	24
	• 00X-026-082	25
	• S25	26
Salix miyabeana	• Verona (Clone) ID 99201-002)	27
	• SX67	28
	• SX64	29
Salix sachalinensis	• SX61	30
Salix dasyclados	• SV1	31
Salix purpurea x Salix miyabeana	• Oneonta (Clone ID 9879)	1
	• Saratoga (Clone ID 99217-023)	2
	• Oneida (Clone ID 9980-005)	3
	• Millbrook (Clone ID 99217-015)	4
Salix sachalinensis x Salix miyabeana	• Cicero (Clone ID 9870-1)	5
	• Marcy (Clone ID 9870-23)	6
	• Canastota (Clone ID 9970-036)	7
	• Sherburne (Clone ID 9871-31)	8
Salix viminalis x Salix miyabeana	• Tully Champion (Clone ID 99202-011)	9
	• Fabius (Clone ID 99202-004)	10
	• Owasco (Clone ID 99207-018)	11
	• Truxton (Clone ID 99207-020)	12
	• 01X-268-015	13
	• Taberg (Clone ID 99202-043)	14
	• 99208-038	15
	• Otisco (Clone ID 99201-007)	16
Unknown	• S365	32
	• Hotel	33
	• Charly	34
	• India	35
	• Juliet	36

The willow lines were provided as clonal material by the willow group at the State University of New York (SUNY) and some from willow programs in Sweden. The plantation was established on the U of S Research Farm on campus in Saskatoon in 2007 as double-row plantings (0.75 m between rows and 1.5 m between lines and 0.6 m between trees within the row). Each row had 13 uniform plants. Leaves for DNA extractions and flow cytometry were collected from the first plant of the first row for each line.

Material from line SX67 could not be obtained for flow cytometry analysis. The lines Hotel and 00X-026-082 were not considered in the ISSR analysis because of poor DNA. Material of *Salix viminalis* was obtained from the Canadian Wood Fibre Centre, Edmonton, and used in the RAPD analysis.

3-3-2 Flow cytometry

Two young leaves of each line were collected, put in between moist paper towels and kept in separated and labelled zip-lock bags. The package was sent to the flow cytometry laboratory at the Benaroya Research Institute,Virginia Mason, (Washington), where the nuclear DNA content was measured using propidium stained nuclei with a BD FACScan flow cytometer. Chicken red blood cell nuclei (2.50Pg/2C) were used as a standard for comparison.

3-3-3 DNA extraction

Young leaves of each willow line were collected and DNA was extracted. Two DNA extraction procedures were tested. The CTAB (cetyltrimetylammonium bromide)

based extraction procedure with slight modifications of the protocol developed by Procunier et al., (1990) (Appendix1) and the ZR Plant/Seed DNA extraction kit^{TM} (Zymo Research, Burlington), following the manufacturer's procedure.

3-3-4 ISSR primers

Thirty-five ISSR primers from the University of British Columbia (UBC) biotechnology laboratory were used (Appendix 2). Primers were 16 to 18 base pairs long and prepared at a concentration of 5µM, and stored at -20ºC.

3-3-5 RAPD primers

Thirty-five long RAPD (15 base pairs) synthesised by Invitrogen, Canada, and 55 (10 base pairs) primers (Operon technologies, California) (Appendix 3) were tested. All primers were diluted to a final concentration of 5µM and kept at -20ºC.

3-3-6 DNA amplification

A GeneAmp PCR System 9700 was used for DNA amlification. The PCR was performed in a 25µl reaction containing 16.3µl water, 2.5µl buffer, 1µl of a 25mM $MgCl_2$, 2µl of a 5mM dNTP solution, 2µl of 5µM primer solution, 0.2µl of polymerase and 1µl DNA template. For RAPD, the PCR was performed under the following cycling conditions: 94ºC (3 min) for initial denaturation, followed by 35 cycles of 94ºC (45 sec) denaturation, 37ºC (45sec) annealing and 72ºC (1min) extension. A 5min final extension ended each PCR reaction. For ISSRs, the same protocol was used but the annealing temperature varied among primers (Appendix 2). All reactions were set on ice prior to

amplification and were prepared in two steps: first, all ingredients except for the DNA template were pipetted into a 1.5ml micro-tube, then 24µl of this mixture was pipetted into 0.6ml micro-tube, and 1µl of DNA template added. The buffer, the $MgCl_2$ and Taq were used as supplied by Invitrogen, Canada.

3-3-7 Separation of amplified fragments

The amplified bands were separated by electrophoresis on a 1.25% agarose gel (Ultrapure DNA grade agarose, BioRad) in a tris-borate- EDTA (Ethylene-Diamine-Tetraacetic Acid) (0.5X TBE (tris base, boric acid and EDTA) buffer. The gels were pre-stained at 0.00001% with a 0.5µg/ml ethidium bromide solution. Five micro-litres of DNA gel loading buffer were added to the 25µl PCR reaction mixture. Gels were photographed on a Ultraviolet (UV) transluminator with Polaroid ^{TM} film. A 1 kb molecular weight marker (Invitrogen, Canada) was run alongside the samples for size assessment.

3-3-8 Scoring and data analysis

Cluster analysis was performed using the Numeric Taxonomic and Multivariate Analysis System (NTSYS) version 2.2. Banding patterns were scored from photographic negatives of gels. The presence of a band was scored as (1), the absence as (0), and missing data as (999) as required by NTSYS software. For each primer, only strong, reproducible and polymorphic bands were scored. Dendrogams were constructed using the Unweighted Pair Group Method (UPGMA) and the Sequential, Agglomerative, Hierarchical, and Nested Clustering method (SAHN).

3-4 Results

3-4-1 DNA extraction

CTAB DNA extraction from willow leaves was successful, but relatively large amounts of foliar pigments were also extracted, which could not be removed, especially for lines of *S. eriocephala*, *S. dasyclados*, Indiana and S365.

The ZR Plant/Seed DNA kit yielded small volumes of DNA in solution (50µl), but the DNA was of better quality. No pigment coloration was noticed. The extraction procedure was simple and faster (15min) and yielded 8 to 10µg of DNA from 0.15 g of initial leaf material. DNA used in this project was extracted using the ZR Plant/Seed DNA kit.

3-4-2 Flow cytometry

DNA amounts (pg/2C nucleus) varied from 1.14 to 3 (Table 3.2). There was some variation among lines belonging to the same species, and among hybrids from the same parents (Table 3.2).

Table 3.2 Nuclear DNA content of the willow lines used in the study

Species/Hybrid	Number of lines	Range in DNA amount 2C (pg)
S. miyabeana	2	2.03-2.07
S. sachalinensis	1	2.06
S. purpurea	5	1.25-1.35
S. erocephala	4	1.14-1.32
S. dasyclados	1	1.31
S. viminalis x S. miyabeana	8	1.59-1.84
S. sachalinensis x S. miyabeana	4	1.99-2.21
S. purpurea x S. miyabeana	4	1.78-1.88
Hotel	1	1.31
Juliet	1	1.22
Charly	1	2.77
India	1	3.00
S365	1	1.99

3-4-3 ISSR analysis

Of the 35 ISSR primers tested, 19 produced multiple banding patterns from the entire set of the willow lines. The number of scorable bands per primer varied between 3 and 6. A total of 70 bands were produced. Of these, 35 were monomorphic and 35 were polymorphic. The results of some primers that produced polymorphic bands are shown in Figures 3.1-3.4. Lines Hotel (33) and 00X-032-094 (25) had poor DNA quality, and did not produce band. A 1 kb molecular weight marker was run alongside the samples for size assessment.

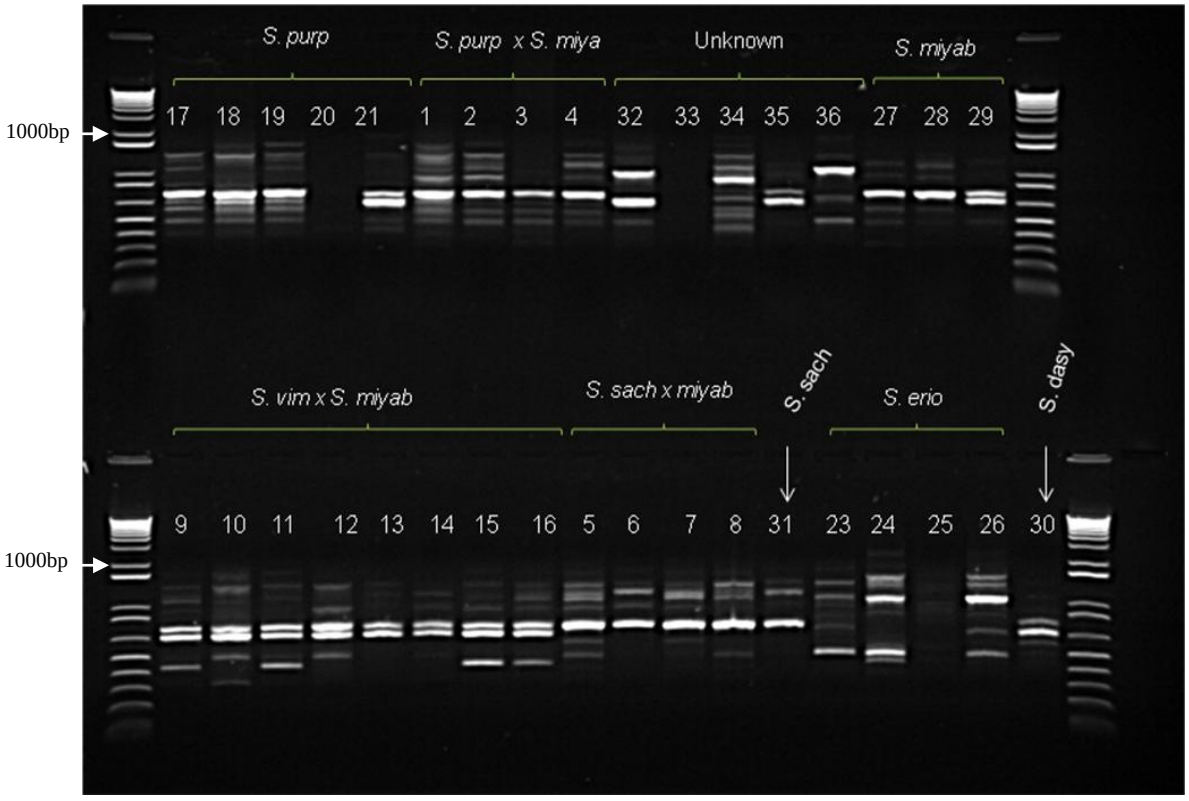


Figure 3.1: Banding patterns produced by PCR amplification of willow lines with primer ISSR 825. Line Onondaga (20) did not produce any bands.

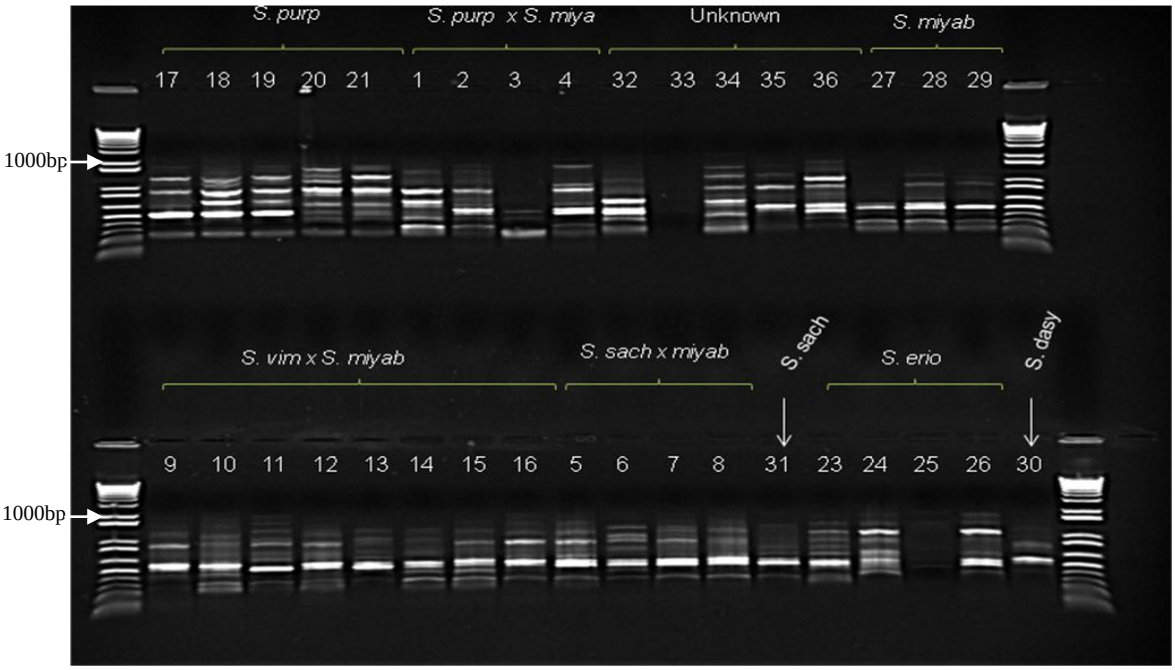


Figure 3.2: Banding pattern produced by PCR amplification of willow lines with primer ISSR 834.

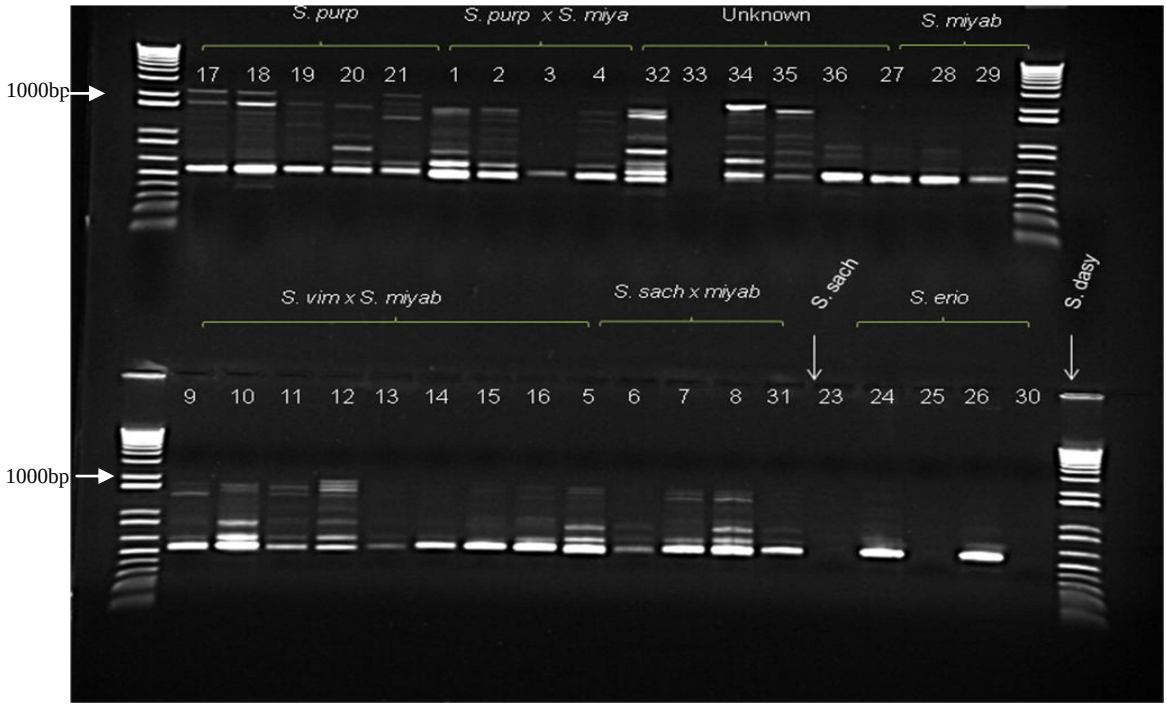


Figure 3.3: Banding pattern produced by PCR amplification of willow lines with primer ISSR 843. Lines 9837-77 (23) and SVI (30) did not produce any bands.

On the ISSR dendrogram (Figure 3.5), lines of the same species were grouped together except for line 9837-77 (*S. eriocephala)* which was grouped with lines of *S. miyabeana*. The line S365 was grouped with lines of *S. purpurea.* The line India and the only line of *S. dasyclados* (SVI) were grouped together. The line Charly showed the lowest similarity (0.56) and was distant from other lines. The line Juliet was grouped with lines of *S. eriocephala*. Inter-specific hybrids were grouped in the middle of the dendrogam, and clustered based on the parents used.

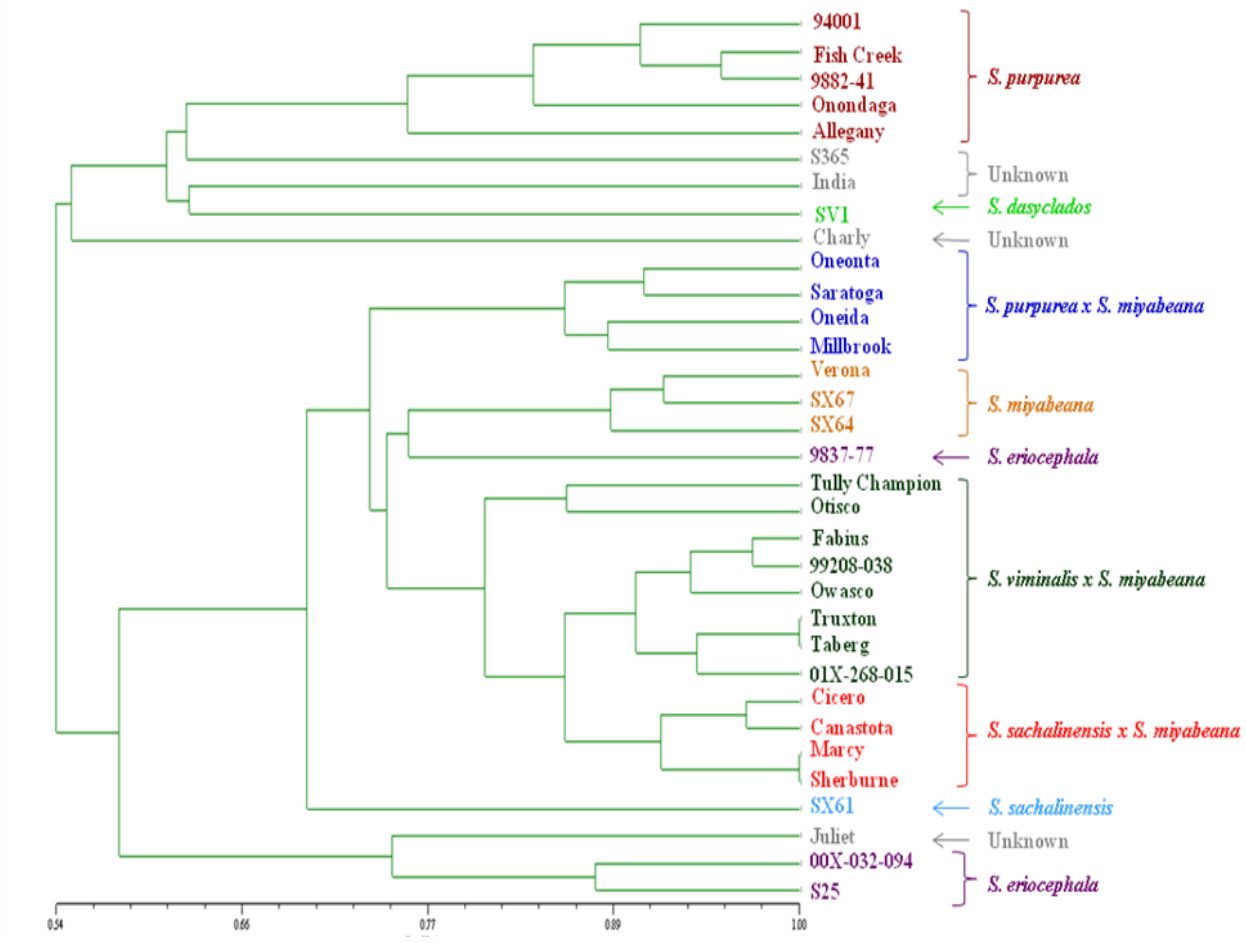


Figure 3.4: Dendrogram illustrating genetic similarities among investigated willow species and hybrid lines generated by UPGMA cluster analysis estimated from 35 ISSR polymorphic bands produced by 19 ISSR primers. The lines Hotel, 00X-026-082, and *S. viminalis* were not included in this experiment.

3-4-4 RAPD analysis

Each of the RAPD primers was pre-screened with a single line of each species. Over 90 primers were tested, sixty primers were chosen to be applied to the entire set of lines; Only Primers showing three or more bands were used to amplify the rest of the lines. The number of scorable bands per primer varied between 3 and 10. A total of 197 scorable bands were produced. Of these, 98 were monomorphic and the remaining 99 were polymorphic and scored for cluster analysis. The lines Hotel, 00X-026-082 and *S. viminalis* were included in this experiment, although not used in the ISSR analysis. An example of RAPD banding patterns is shown (Figure 3.6).

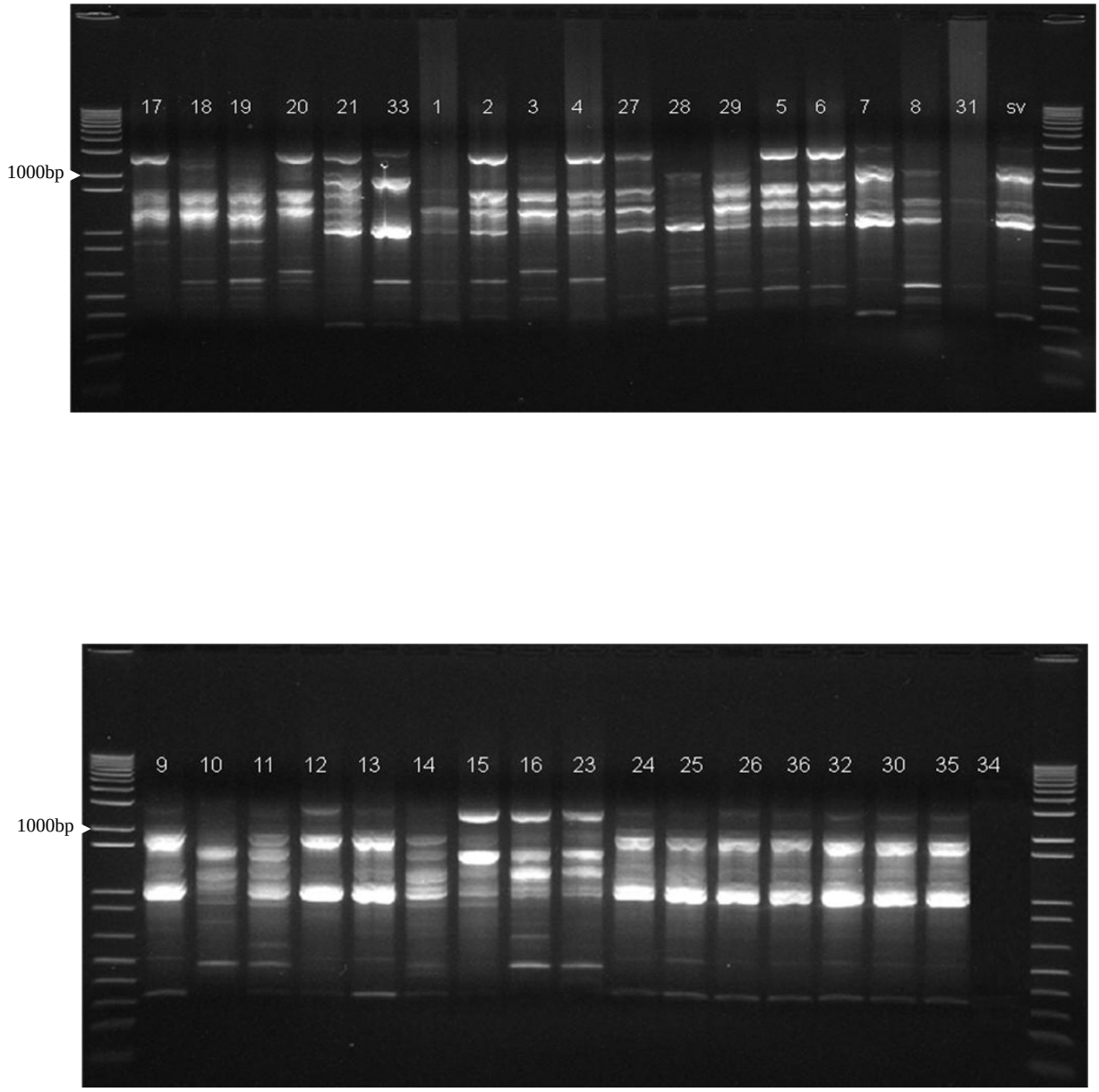


Figure 3.5: Banding patterns produced by PCR amplification of willow lines with RAPD primer CMG. (Lines SX61 (31) and Charly (34) did not produce bands.

The RAPD dendrogram was very similar to the ISSR dedrogram (Figure 3.5), with hybrid lines in the middle, and lines of the same species grouped in clusters. However, some differences were observed. The line Hotel was grouped with lines of *S. purpurea*, and the line 9837-77 was grouped with other lines of *S. eriocephala*. The only line of *S. viminalis* was close to hybrids *S. viminalis x S. miyabeana*, and the only line of *S. sachalinensis* was grouped with *S. sachalinensis x S. miyabeana* hybrids.

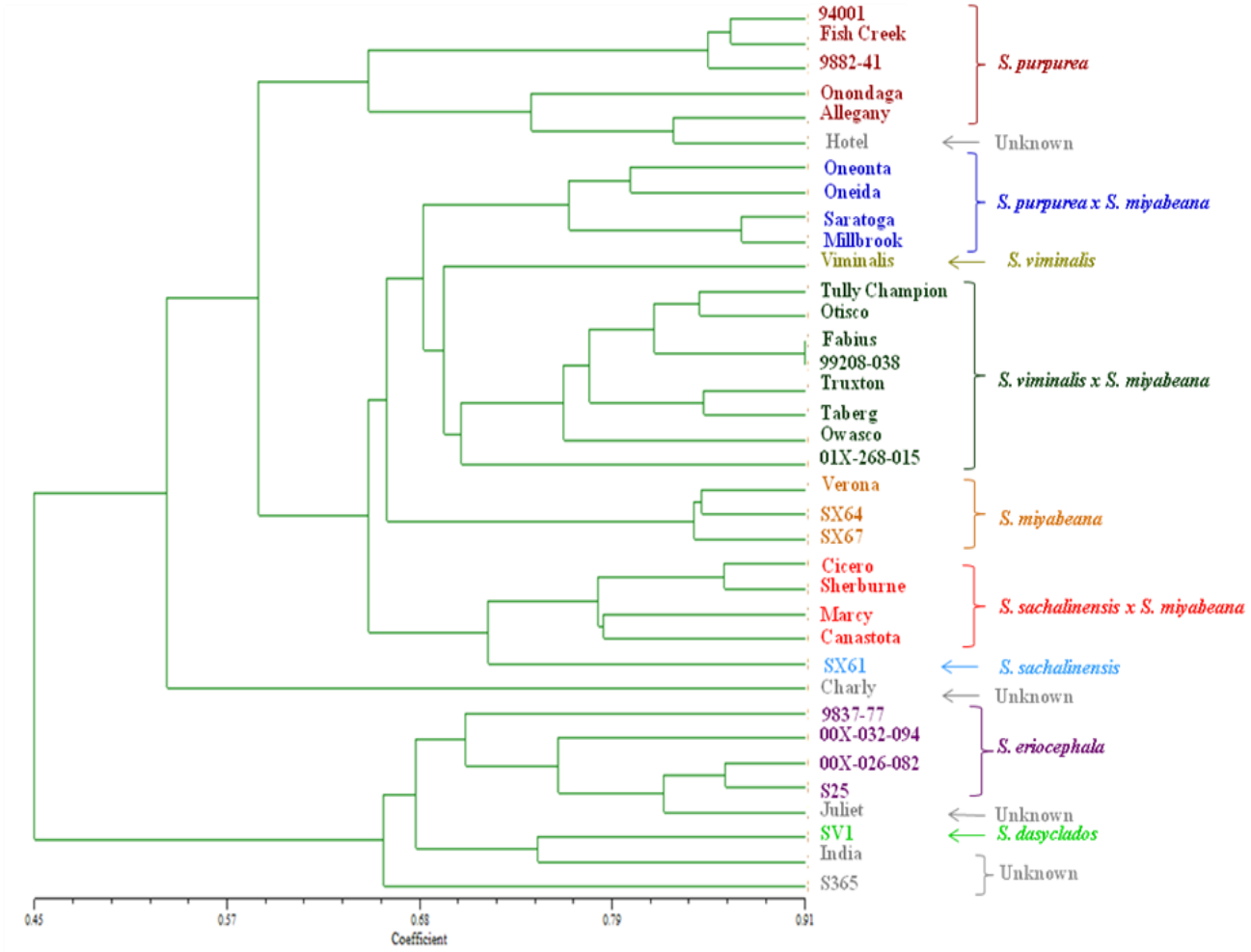


Figure 3.6: Dendrogram illustrating genetic similarities between willow species and hybrids generated by UPGMA cluster analysis (NTSYS PC v2.2) calculated from 99 RAPD markers produced by 60 RAPD primers.

3-5 Discussion and conclusions

The objective of this series of experiments was to evaluate the nuclear DNA content and the use of ISSR and RAPD marker techniques to assess genetic relationships among the set of biomass willow lines being investigated by the Center for Northern Agroforestery and Aforestation in Saskatchewan.

Flow cytometry has become widely used for the measurement of the nuclear DNA content and ploidy screening in plants, and has proved to be rapid and more efficient than the previous methods including Feulgen microspectrophotometry and DNA image densitometry (Doležel and Bartos, 2005). The only previous estimation of DNA content in willow was performed by Thibault (1998). He reported 2C DNA contents in diploids, triploids and tetraploids as shown in Table 3.3.

Table 3.3 Comparison between estimated 2C nuclear DNA amounts of material; used in this study and the published 2C nuclear DNA amounts (Thibault, 1998)

Range in DNA amount pg/2C (Thibault, 1998)	**Estimated range in DNA amount pg/2C (this study)**	**Ploidy**
0.76 to 0.98	/	diploid
1.17-1.23	1.14 -1.35	triploid
1.62-1.80	1.59-2.21	tetraploid
/	2.77-3	hexaploid

Thus, all lines used in this study appeared to be polyploids. The nuclear 2C DNA value ranges tended to be wider than those reported by Thibault (1998), particularly at the higher end. Ploidy levels of the material used in this study were estimated to vary from

triploids to hexaploid (Table 3.4). The hybrids *S. viminalis x S. miyabeana*, *S. purpurea x S. miyabeana* and *S. sachalinensis x S. miyabeana* were tetraploids (4x/4x crosses) (Table 3.4).

Table 3.4 Estimated ploidy level of the willow lines used in the study

Species/Hybrid	**Estimated ploidy level**
S. miyabeana	4x
S. sachalinensis	4x
S. purpurea	3x
S. erocephala	3x
S. dasyclados	3x
S. viminalis x S. miyabeana	4x
S. sachalinensis x S. miyabeana	4x
S. purpurea x S. miyabeana	4x
Hotel	3x
Juliet	3x
Charly	6x
India	6x
S365	4x

Thibault (1998) reported *S. purpurea* as a diploid, with a 2C value of 0.94pg. The *S. purpurea* lines used in this study had higher DNA values than Thibault (1998) and thus appeared to be triploids, with 2C values ranging between 1.25-1.35pg. No 2C nuclear DNA values for other *Salix* species were reported by Bennett and Leitch (2005). Gaiser (1930) reported unequal chromosome pairing and frequent production of trivalents and quadrivalents during meiosis in some *Salix* species. This could explain the observed intraspecific variation of the 2C DNA values. Sinoto, (1929) reported the haploid

chromosome number of *S. sachalinensis* to be n=19, but mentioned the existence of n=24 haploid cells in a form of *S. sachalinensis* from Hokkaido. No information on chromosome number of *S. miyabeana*, and *S. eriocephala* was found in the literature. Intra-specific variation in genome size has been reported in many plant species (Bennett, 1985). The results of this experiment confirmed the variability of 2C nuclear DNA values and ploidy levels both within and between *Salix* species (Hakansson, 1955; Suda and Argus, 1968; Thibault, 1998). All hybrid lines were tetraploids from crosses between tetraploid parents.

Both RAPD and ISSR dendrograms showed the same clusters with some minor differences. The ISSR dendrogram grouped line 9377-77 with lines of *S. miyabeana*, whereas the RAPD dendrogram, grouped the same line along with other lines of *S. eriocephala*. This difference could be due to scoring errors or to the reduced number of scoring data (35) used to construct the ISSR dendrogram. The RAPD dendrogram produced a slightly better separation of clusters with a higher number of scoring data (99).

The line Hotel was clustered with other lines of *S. purpurea*, line Juliet was clustered with lines of *S. eriocephala*, and lines S365 and India were in both RAPD and ISSR studies grouped with the only line of *S. dasyclados* (SVI).

ISSR primers produced fewer bands (3 to 6), which appeared stronger and reproducible. RAPD primers produced more bands (5-9), but most of them appeared weak and less reproducible compared to ISSRs.

3-5-1 Markers for specific lines

All of the hybrid lines investigated had *S. miyabeana* as a common parent. From the banding pattern developed with primer ISSR825, a strong band of 500bp was specific to hybrid lines *S. viminalis x S. miyabeana*, and could be used as a marker to identify them from other hybrid lines (*S. purpurea x S. miyabeana* and *S. sachalinensis x S. miyabeana*) as illustrated in Figure 3.8.

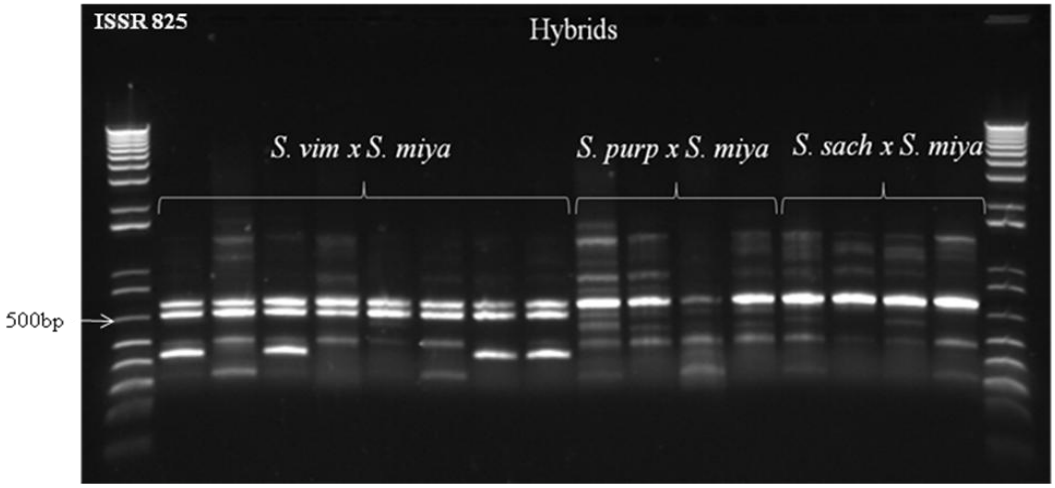
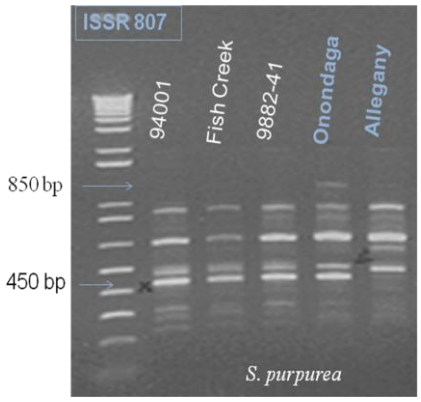
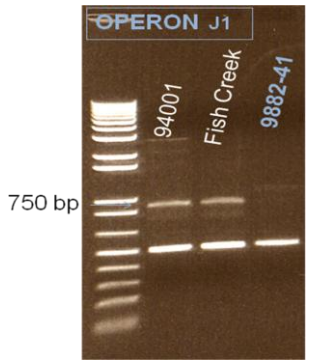


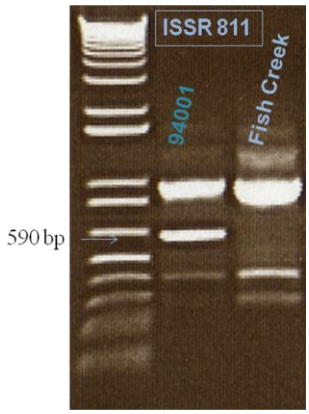
Figure 3.7: Band 500bp identifies hybrid lines *S. viminalis x S. miyaneana* from other hybrid lines used in the program.1kb ladder is used to assess band sizes.

Similarly, strong reproducible and polymorphic ISSR and RAPD bands were identified that could be used in combination to fingerprint individual lines. Key is proposed for identification among lines of *S. purpurea* (5), among hybrids of *S. purpurea x S. miyabeana* (4), and among hybrids of *S. sachalinenesis x S. miyabeana* and the only line of *S. sachalinensis* (5).

Identification key for lines of *S. purpurea*







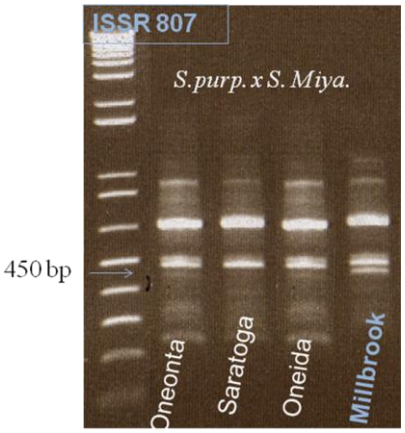
ISSR 807

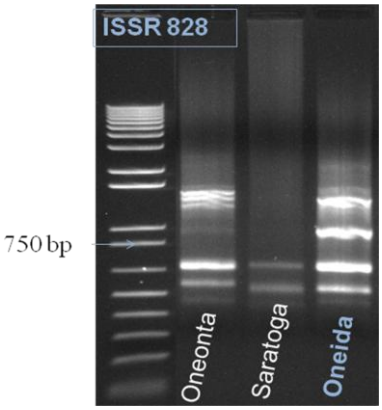
1-a if band 450 bp present, then go to ISSR 807-2-a

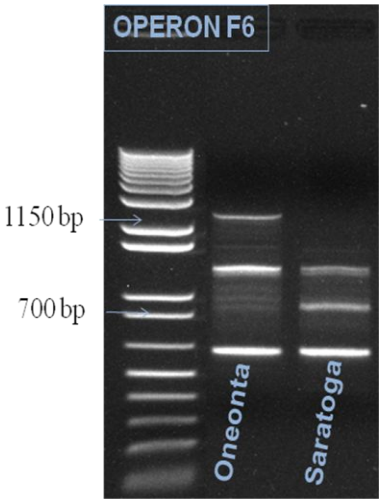
1-b if absent, then **Allegany**

2-a if band 850 bp present, then **Onondaga**

Identification key for hybrid lines of *Salix purpurea x Salix miyabeana*







ISSR 807

1-a if band 450 bp present, then **Millbrook**

1-b if absent, go to ISSR 828

ISSR 828

1-a if band 750 present, then **Oneida**

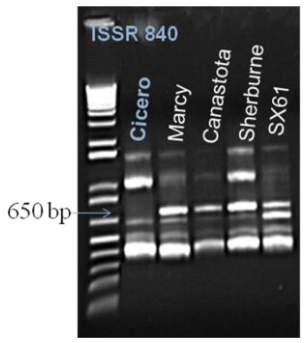
1-b if absent, then go to Operon F6

Operon F6

1-a if band 1150 present, then **Oneonta**

1-b if absent, then **Saratoga**

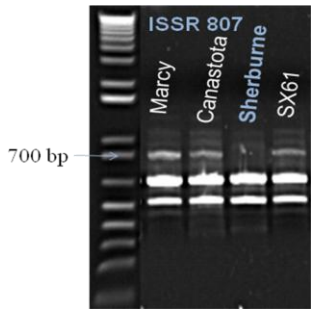
Identification key for hybrid lines of *S. sachalinensis x S. miyabeana* and the only line of *S. sachalinensis* (SX61)



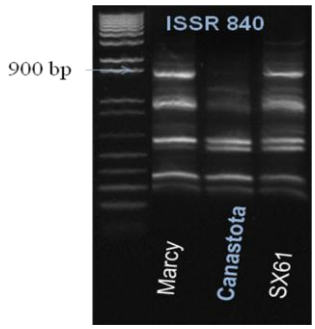
ISSR 840

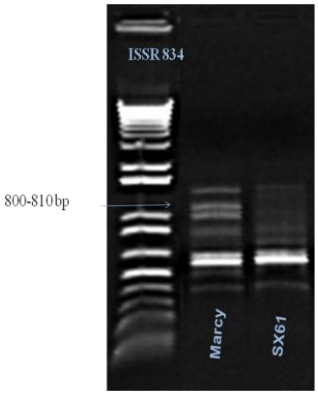
1-a if band 650 bp present, then go to ISSR 807

1-b if absent, then **Cicero**



ISSR 807





4-THE USE OF 18S-5.8S-28S INTERNAL TRANSCRIBED SPACER (ITS) FOR SPECIES IDENTIFICATION IN THE GENUS *Salix*

4-1 Abstract

Given that morphological identification of willow species used in the project is difficult, the DNA region coding for ribosomal RNA including the entire 5.8S RNA region and the internal transcribed spacers (ITS1 and ITS2) was amplified and sequenced to assess sequence homology between five *Salix* species (*Salix purpurea*, *Salix eriocephala*, *Salix sachalinensis*, and *Salix dasyclados*). The total length of the amplified region was 601bp, with the ITS1, 5.8 S and ITS2 being 223, 163, and 215bp respectively. Intra- and inter-species SNPs were observed, 6 within ITS1, and 3 within ITS2. No polymorphisms were found in the 5.8S gene. The low rate of variation within the sequenced ITS fragment between species supports the monophyly of the five species involved in this study, and confirms their belonging to the subgenus *Caprisalix*. SCAR primers were designed from species specific polymorphic nucleotides and applied to the willow collection to test their use for species identification. A species identification key based on SNPs is proposed.

4-2 Introduction

Nuclear rRNA genes provide markers for phylogeny at a variety of taxonomic levels (Soltis and Kuzoff, 1993). The 18S-5.8S-28S tandem repeats are located in the nucleolar organizing region of satellited chromosomes. Each repeat unit consists of a single transcribed region for the 18S, 5.8S and 28S ribosomal RNAs, two small internal transcribed spacers (ITS1 and ITS2) and a large external non transcribed inter-genic spacer (IGS) (Flavell, 1986). ITS sequences are easy to amplify, even with small quantities of DNA, and show a high level of variation even between closely related species (Chen et al., 2001; Baldwin, 1992). DNA coding for the 18S, 5.8S, and 28S rRNAs are highly conserved. The spacers undergo concerted evolution where they evolve relatively together by accumulating mutations, and provide a source of polymorphisms between species and genera (Downie and Downie, 1996).

In this experiment, the internal transcribed spacers (ITS1 and ITS2) were investigated for length and DNA sequence variation among the willow species (*Salix purpurea*, *Salix miyabeana*, *Salix eriocephala*, *Salix dasyclados*, *Salix sachalinensis*) used in this project. SCAR markers were developed from species-specific polymorphisms.

4-3 Materials and methods

4-3-1 Plant material

Five willow lines were randomly chosen from among the biomass willow collection grown by the Center for Northern Agroforrestery and Afforestation of the University of Saskatchewan for this experiment (Table 4.1), each representing one species used in the project.

Table 4.1 Selected willow lines for ITS analysis

Species	*S. purpurea*	*S. eriocephala*	*S. miyabeana*	*S. sachalinensis*	*S. dasyclados*
Clone ID	94001	9837-77	SX64	SX61	SVI

4-3-2 Primer design

Primers for the regions of the 18S, 28S genes that flank the ITS1/5.8S/ITS2 regions (Figure 4.1) were designed from partial sequences of a number of 18S and 28S sequences of the genus *Populus* in GenBank, and synthesised by Invitrogen Canada (Burlington, Ontario). Prior to use, the primers were diluted in sterile distilled water to a final concentration of 5µM, and stored at -20ºC.

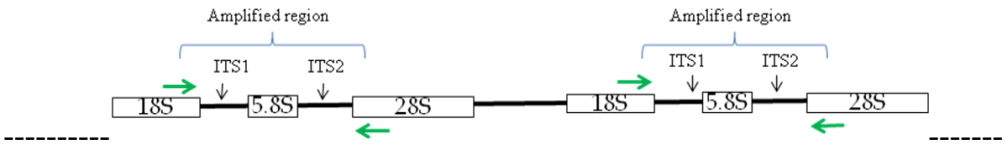


Figure 4.1: Approximate location of primers for the amplification of ITS regions

4-3-3 DNA extraction

Young leaves of the selected lines were collected. One hundred and fifty milligram of leaf material was used for DNA extraction, following the ZR Plant/Seed DNA extraction kit™ (Zymo Research, Burlington).

4-3-4 DNA amplification and gel electrophoresis

DNA amplification was performed using a GeneAmp PCR System 9700. Each PCR was performed in a 25µl reaction containing 16.3µl of water, 2.5µl buffer, 1µl $MgCl_2$, 1µl of the 5µM 18S primer solution, 1µl of the 5µM 28S primer solution, 2µl of a 5mM dNTP solution, 0.2 µl of Taq polymerase (Invitrogen Canada) and 1µl of the DNA template. The buffer, the $MgCl_2$ and Taq were used as supplied by Invitrogen, Canada. The thermo-cycler was set for the following cycling conditions: 94º C (3min) for initial denaturation, followed by 35 cycles of 94ºC (45sec) denaturation, 65º C (45sec) annealing and 72º C (1min) extension. Each PCR reaction ended with a 5min final extension period. The amplification reaction was repeated to check for reproducibility. From the 25µl of each PCR reaction, 19µl were used for electrophoresis and 6µl were retained for cloning and sequencing.

Amplified fragments were separated by gel electrophoresis on a 1% agarose gel in tris borate EDTA (0.5X TBE) buffer, and visualised by pre-staining with ethidium bromide (0.1µg /ml). Five micro-litres of DNA gel loading buffer were added to each reaction before loading the gel. A 1kb molecular weight marker was run along the side of the samples for size assessment. Stained ITS fragments were viewed under UV light.

4-3-5 Cloning and sequencing

Two micro-litres of the ITS fragment PCR reaction were used for cloning. The ITS fragments were ligated into pCR®4-TOPO® vector plasmid (Invitrogen, Canada). The recombinant plasmid vectors were transformed into One Shot ® TOPO10 competent *E.coli* cells and grown overnight on LB plates containing 100µl/ml of a 50 mg/ml ampicillin solution. Ten isolated colonies were aseptically picked and grown separately on 5ml LB broth (Gibco) medium containing 5µl of a 50mg/ml ampicillin solution.

To confirm the presence of the appropriate insert, PCR reactions were carried out using 1µl of each colony as template. The GeneAmp PCR System 9700 was used. Each PCR was performed in a 25µl reaction containing 16.3µl of water, 2.5µl buffer, 1µl $MgCl_2$, 1µl each of the primers used to initially create the fragment, 2µl of dNTP, 0.2µl of Taq polymerase (Invitrogen, Canada), and 1µl of the bacterial culture. The amplification program used for PCR was the same as that initially used to produce the fragments of interest.

All reactions were analysed on a 1% agarose gel in tris borate EDTA (0.5X TBE) buffer, and visualised by pre-staining with ethidium bromide (0.1µg /ml). Five micro-litres of DNA gel loading buffer were added to each reaction before loading the gel. A 1kb molecular weight marker was run along side of the samples for size assessment, and the bands were viewed under UV light.

Colonies with the expected insert size were purified following the QIAprep ® Miniprep kit. Extracted recombinant vectors were sent for sequencing at the Plant Biotechnology Institute (PBI) in Saskatoon (Canada). The whole experiment was

repeated once for each line to produce up to 20 sequences per line. DNAMAN software (Lynnon Biosoff, 1994-1997) was used for sequence alignment.

4-3-6 Species-specific SCAR markers

Primers were designed based on species-specific SNP sites in an attempt to develop species-specific SCAR markers (Table 4.2). These were used in combination with the 28S primer (CCGCCTGACCTGGGGT) as reverse primer. PCR reactions were performed under the following cycling conditions (94º C (3min) for initial denaturation, followed by 35 cycles of 94º C (0.45min) denaturation, (0.45min) annealing at variable temperatures (Table 4.2), 72º C (1min) extension. Each PCR reaction ended with a 5min final extension. For each primer, the annealing temperature was progressively increased in separate PCR reactions to improve the specificity of the amplified product in the target species (Table 4.2).

Table 4.2 Primer sequences designed from informative species-specific SNP sites. (Bolded bases represent nucleotide specific sites)

SNP	**Target species**	**Forward primer**	**Tested Annealing temperatures**
7	*S. purpurea*	AAGGATCATTGTCGAA**G**	62ºC, 63ºC, 64ºC
56	*S. eriocephala*	GCATGACAAGCTGGGC**C**	67ºC, 69ºC, 70ºC
172	*S. dasyclados*	ATTGAGTACTAGGAGC**C**	67ºC, 68ºC, 70ºC
180-181	*S. sachalinensis*	GGAGCACGCCCTC**TA**	67ºC, 68ºC

4-4 Results

The PCR amplification produced monomorphic bands of approximately 638 bp (Figure 4.2).

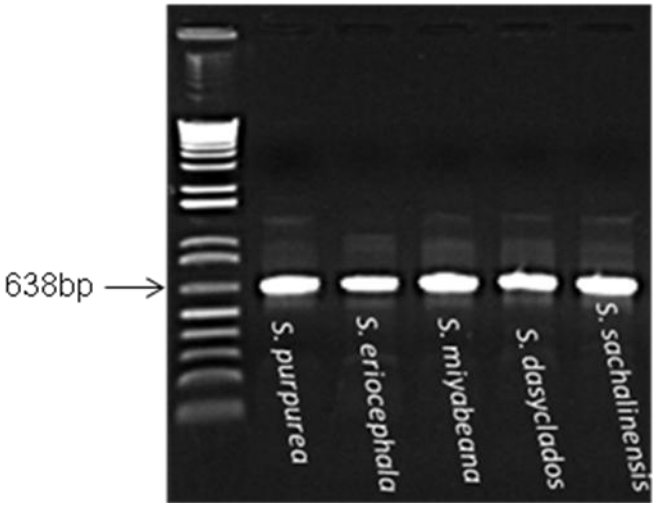


Figure 4.2: PCR amplification of the ITS1-5.8S-ITS2 DNA region produced monomorphic bands.

After cloning of the inserts and re-amplification with the primers used originally, some clones did not carry the insert. On average, 79% of the *E. coli* colonies had an insert of the expected size (Table 4.3).

Table 4.3 Percentage *E. coli* colonies with ITS insertions.

Species	**Number of colonies**	**Colonies with insert**	**Percentage with inserts**
S. purpurea (94001)	20	15	75
S. eriocephala (9837-77)	20	16	80
S. dasyclados (SVI)	20	16	80
S. miyabeana (SX64)	20	17	85
S. sachalinensis (SX61)	20	15	75
Totals/Average	**100**	**79**	**79**

4-4-1 Sequence alignment and nucleotide polymorphisms

After the removal of primer sequences, amplified fragments sized 601bp. No size variation was noticed either within or between species. The ITS1, 5.8S, and ITS2 regions were identified by comparing with the *S. purpurea*, and *S. dasyclados* ITS1, 5.8S, ITS2 sequences deposited in GenBank (Leskinen and Alstrom, 1999; Hardig and Brunsfeld, 2008), and were respectively 223, 163 and 215bp. Both intra- and inter-species single nucleotide polymorphisms were observed.

4-4-1-1 Intra-clonal SNPs

Out of 16 *S. dasyclados* ITS clones, 2 intra-species SNPs at sites 484 and 489 were found. Ten clones had a G and a T, while the 6 remaining had an A and a C respectively (Figure 4.3). Out of 15 *S. sachalinensis* ITS clones, 2 intra-species SNPs

were found at positions 180 and 181. Nine clones had a G and T, while 6 had a T and A (Figure 4.4).

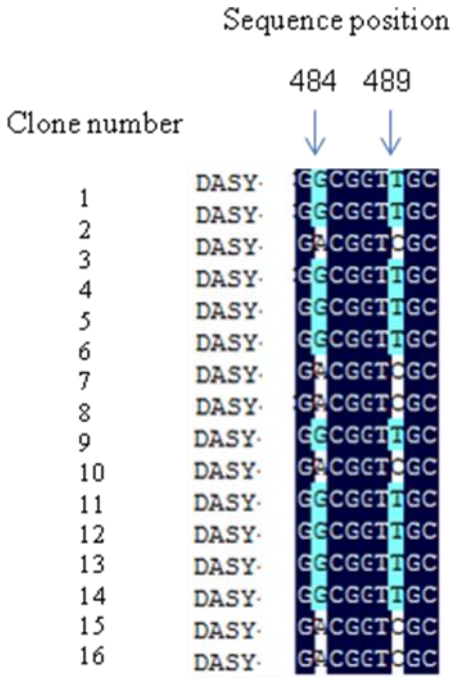


Figure 4.3: 16 clones of segment of ITS fragment showing intra-clonal SNPs in *S. dasyclados*

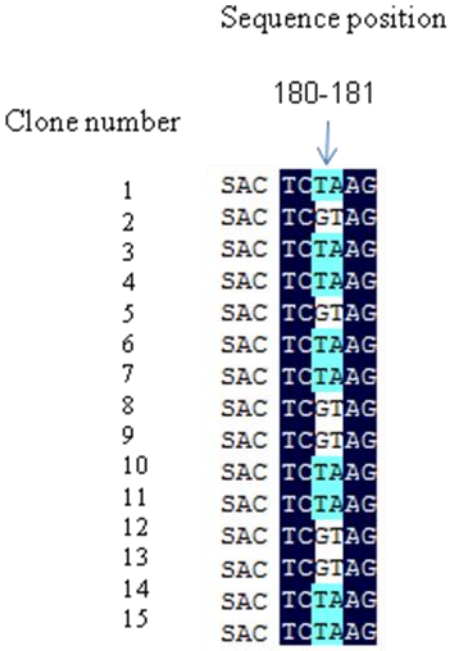


Figure 4.4: 15 clones of segment of ITS fragment showing intra-clonal SNPs in *S. sachalinensis*

These intra-species SNPs led to the identification of two consensuses ITS sequences for *S. dasyclados* (S. dasy1 and S. dasy2) and two for *S. sachalinensis* (S. sacha1 and S. sacha2) (Figures 4.5- 4.7). No intra-species SNP were recorded among ITS clones of *S. purpurea*, *S. miyabeana* and *S. eriocephala* and one consensus ITS sequence was consequently identified for each of those species (Figures 4.5-4.7).

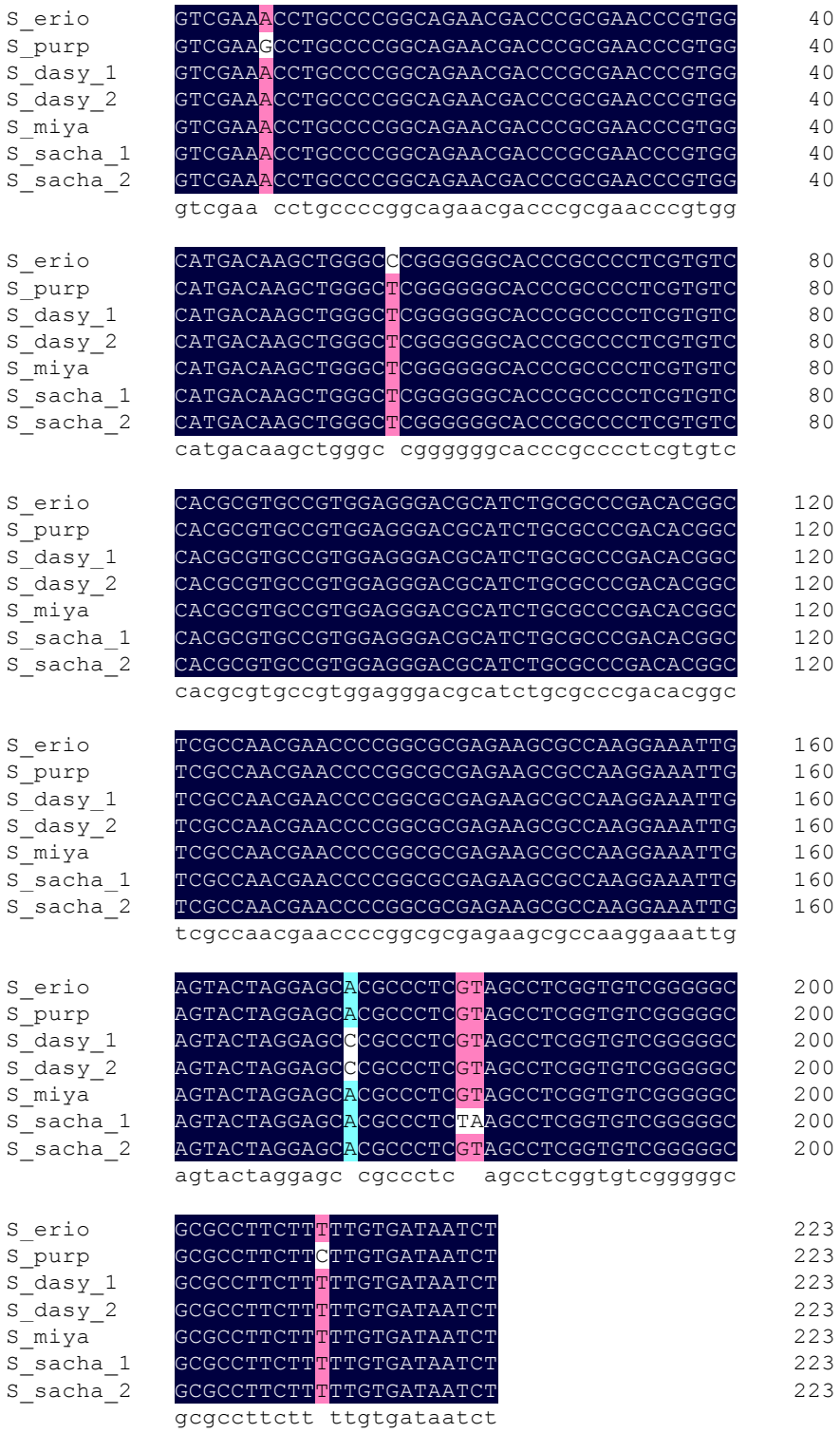


Figure 4.5: ITS1 sequence alignments of consensus fragments of *S. eriocephala*, *S. purpurea*, *S. miyabeana* and the 2 consensus sequences for *S. dasyclados* and *S. sachalinensis* in the 1 to 223 base sequence.

```
S_erio        GAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA    263
S_purp        GAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA    263
S_dasy_1      GAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA    263
S_dasy_2      GAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA    263
S_miya        GAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA    263
S_sacha_1     GAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA    263
S_sacha_2     GAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA    263
              gaacgactctcggcaacggatatctcggctctcgcatcga

S_erio        TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA    303
S_purp        TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA    303
S_dasy_1      TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA    303
S_dasy_2      TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA    303
S_miya        TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA    303
S_sacha_1     TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA    303
S_sacha_2     TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCA    303
              tgaagaacgtagcgaaatgcgatacttggtgtgaattgca

S_erio        GAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGC    343
S_purp        GAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGC    343
S_dasy_1      GAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGC    343
S_dasy_2      GAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGC    343
S_miya        GAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGC    343
S_sacha_1     GAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGC    343
S_sacha_2     GAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGC    343
              gaatcccgtgaaccatcgagtctttgaacgcaagttgcgc

S_erio        CCGAGGCCTCCTGGTCGAGGGCACGTCTGCCTGGGTGTCA    383
S_purp        CCGAGGCCTCCTGGTCGAGGGCACGTCTGCCTGGGTGTCA    383
S_dasy_1      CCGAGGCCTCCTGGTCGAGGGCACGTCTGCCTGGGTGTCA    383
S_dasy_2      CCGAGGCCTCCTGGTCGAGGGCACGTCTGCCTGGGTGTCA    383
S_miya        CCGAGGCCTCCTGGTCGAGGGCACGTCTGCCTGGGTGTCA    383
S_sacha_1     CCGAGGCCTCCTGGTCGAGGGCACGTCTGCCTGGGTGTCA    383
S_sacha_2     CCGAGGCCTCCTGGTCGAGGGCACGTCTGCCTGGGTGTCA    383
              ccgaggcctcctggtcgagggcacgtctgcctgggtgtca

S_erio        CGC                                         386
S_purp        CGC                                         386
S_dasy_1      CGC                                         386
S_dasy_2      CGC                                         386
S_miya        CGC                                         386
S_sacha_1     CGC                                         386
S_sacha_2     CGC                                         386
              cgc
```

Figure 4.6: 5.8S sequence alignments of consensus fragments of *S. eriocephala*, *S. purpurea*, *S. miyabeana* and the 2 consensus sequences for *S. dasyclados* and *S. sachalinensis* in the 224 to 386 base sequence.

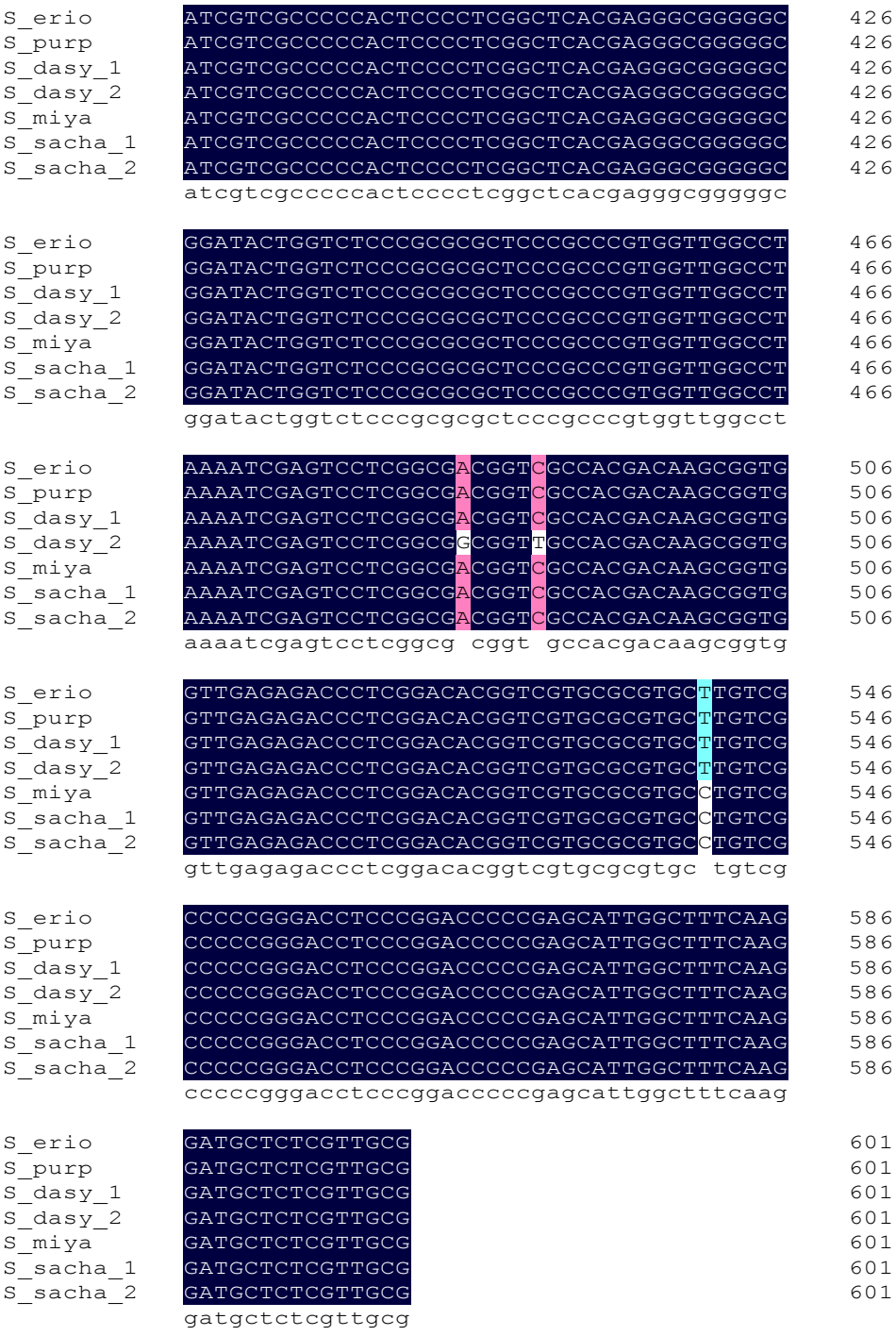


Figure 4.7: ITS2 sequence alignments of consensus fragments of *S. eriocephala*, *S. purpurea*, *S. miyabeana* and the 2 consensus sequences for *S. dasyclados* and *S. sachalinensis* in the 387 to 601 base sequence.

4-4-1-2 Inter-specific SNPs

Sequence alignment of the consensus ITS sequences of the species investigated revealed several species-specific SNPs, 6 within ITS1 and 3 within ITS2 (Table 4.4). No SNP was found within the 5.8S gene.

Table 4.4 Inter-specific ITS1 and ITS2 SNPs between *S.purpurea, S. eriocephala, S. dasyclados, S.miyabeana,* and *S. sachalinensis* (bolded bases represent species-specific SNP sites).

	SNP positions								
	ITS1						**ITS2**		
	7	**56**	**173**	**180**	**181**	**211**	**484**	**489**	**540**
Consensus ITS	A	T	A	G	T	T	A	C	T
S. purpurea	**G**	T	A	G	T	**C**	A	C	T
S. eriocephala	A	**C**	A	G	T	T	A	C	T
S. dasyclados (S. dasy1)	A	T	**C**	G	T	T	A	C	T
S. dasyclados(S. dasy2)	A	T	**C**	G	T	T	**G**	**T**	T
S. sachalinensis (S. sacha1)	A	T	A	**T**	**A**	T	A	C	**C**
S. sachalinensis (S. sacha 2)	A	T	A	G	T	T	A	C	**C**
S. miyabeana	A	T	A	G	T	T	A	C	**C**

S. dasyclados, *S. eriocephala*, and *S. purpurea* had the base T at SNP site 540 (ITS2), while *S. miyabeana* and *S. sachalinensis* had a C. Both ITS sequences of *S. dasyclados* differed from other species having a C at position 173 (ITS1). However, the second ITS sequence of *S. dasyclados* (S. dasy2) was unique among the species studied in having G and T respectively at sites 484 and 489 (ITS2) versus A and C in all others. Whereas both *S. sachalinensis* sequences had a C at position 540 (as did *S. miyabeana*), the first sequence (S.sacha1) was unique to *S. sachalinensis* with a T and A respectively at sites 180 and 181. The unique ITS sequence of *S. purpurea* differed from other sequences with a G and a C respectively on sites 7 and 211 (ITS1). The ITS sequence of *S. eriocephala* differed from others with a C at SNP site 56 (ITS1). The ITS1, 5.8S, and ITS2 sequences

of *S. miyabeana* were very similar to the overall consensus sequence of all five species, but differed with the C at position 540 (Table 4.4).

4-4-2 Species-specific SCAR markers

PCR amplification with primers designed at species-specific SNP sites produced discrete bands which became specific to the targeted species as the optimal annealing temperatures were reached. As the DNA sequence of the region was known, the expected band size produced by such primers could be calculated.

At a 64ºC annealing, primer AAGGATCATTGTCGAA**G** produced the expected 624bp band (major band) and a minor band of 432bp both specific to *S. purpurea* (94001) (Figure 4.8).

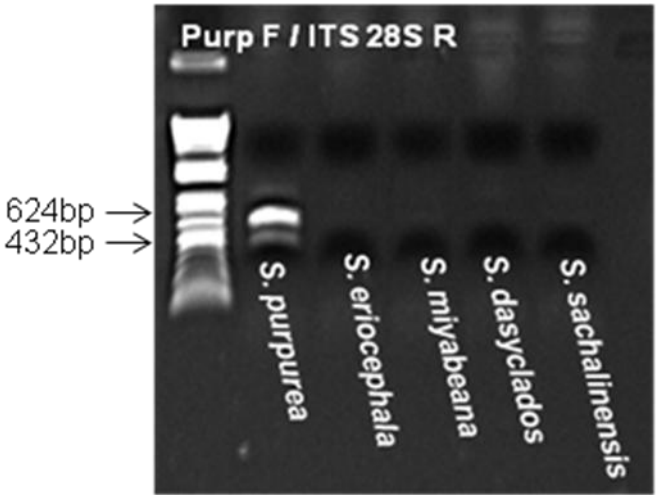


Figure 4.8: PCR amplification with forward primer AAGGATCATTGTCGAA**G** produced a SCAR marker for *S. purpurea* at 64ºC annealing temperature

At a 70ºC annealing temperature, primer GCATGACAAGCTGGGC**C** produced the expected discrete band of 550 bp specific to *S. eriocephala* (9837-77) (Figure 4.9).

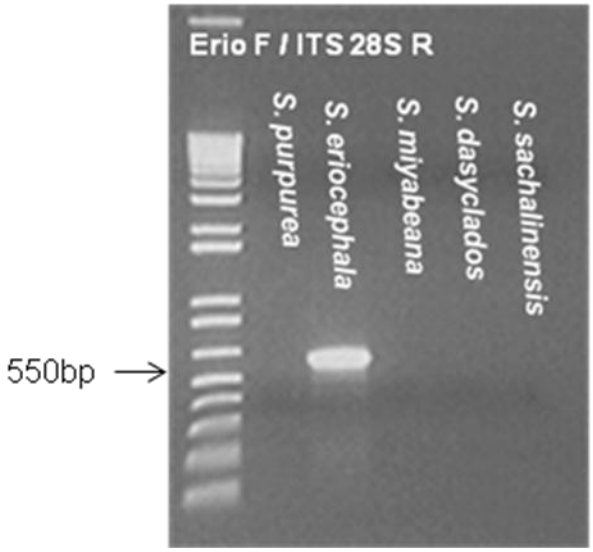


Figure 4.9: PCR amplification with forward primer GCATGACAAGCTGGGC**C** produced a SCAR marker for *S. eriocephala* at 70ºC annealing temperature

At a 68ºC annealing temperature, primer GGAGCACGCCCTC**TA** produced the expected band of 500bp specific to *S. sachalinensis* (SX61) (Figure 4.10).

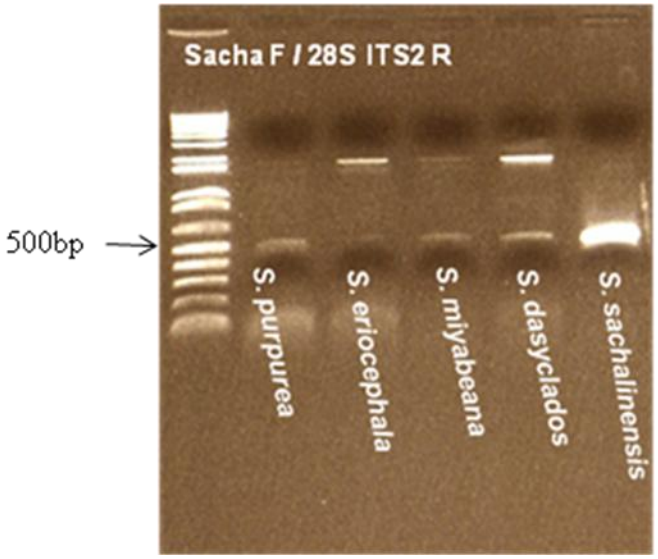


Figure 4.10: PCR amplification with forward primer GGAGCACGCCCTC**TA** produced a SCAR marker for *S. sachalinensis* at 68ºC annealing temperature

PCR reactions with primer ATTGAGTACTAGGAGC**C** designed to target *S dasyclados* did not develop the expected 430bp band size in the target line. However several non specific bands were developed at 65ºC (Figure 4.11).

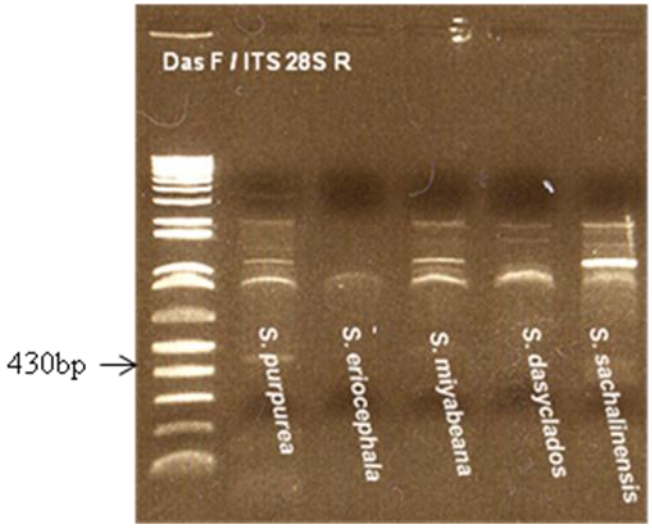


Figure 4.11 PCR amplification with forward primer ATTGAGTACTAGGAGC**C** did not produce the expected 430bp SCAR marker for *S. dasyclados*.

The developed SCAR markers were then applied to all willow lines to test for consistency. At 64ºC, primer AAGGATCATTGTCGAA**G** produced the expected 624bp and the 432bp bands in *S. purpurea* lines, in hybrid lines *S. purpurea x S. miyabeana*, and the line Hotel thought to be *S. purpurea* from ISSR and RAPD analysis. Hybrids of *S. purpurea x S. miyabeana* however produced extra bands to *S. purpurea* lines and line Hotel (Figure 4.12).

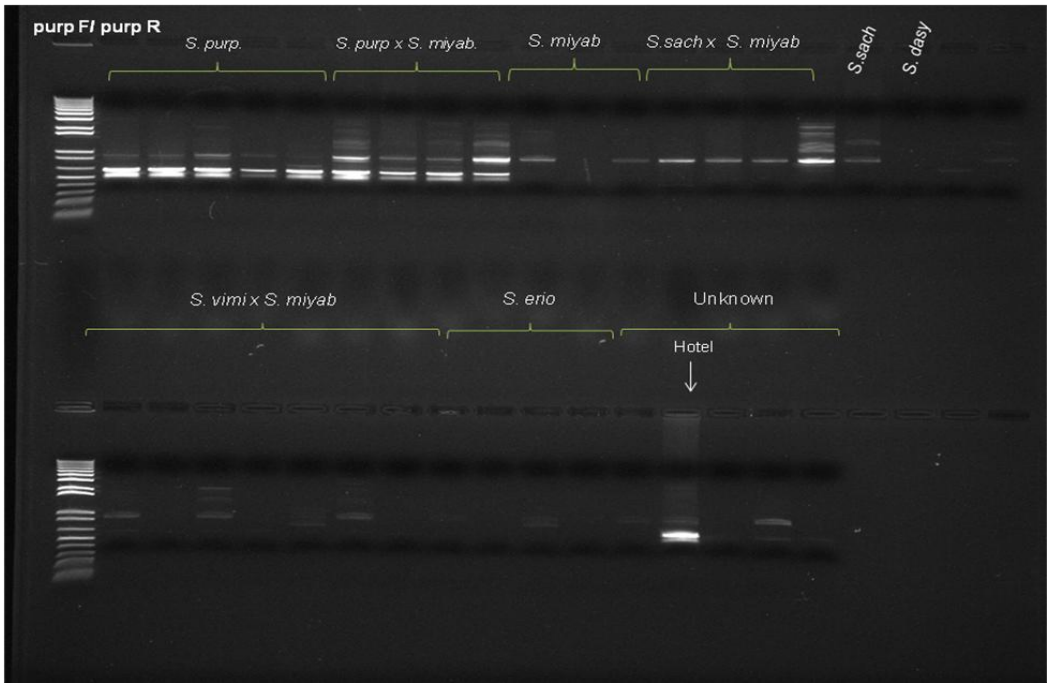


Figure 4.12: Optimal SCAR marker for *S.purpurea* lines, hybrids lines of *S. purpurea x S. miyabeana* and the line Hotel (64ºC annealing)

Primer GGAGCACGCCCTC**TA** produced a strong band of 500 bp in the only line of *S. sachalinensis* (SX61), in hybrids of *S. sachalinensis x S. miyabeana*, and the single line of *S. dasyclados* (SVI). However, some hybrids *S. viminalis x S. miyabeana* also presented weak bands of the expected size (Figure 4.13).

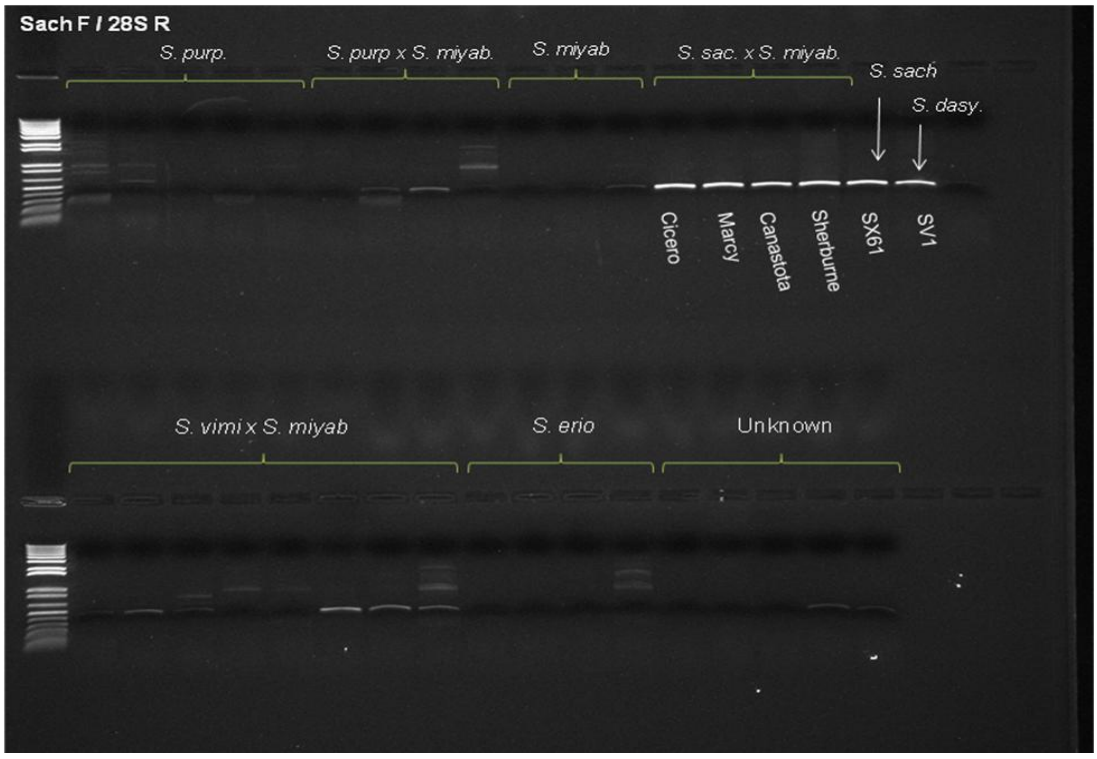


Figure 4.13: SCAR marker for *S. sachalinensis*, hybrids *S. sachalinensis x S. miyabeana* and line SVI (68ºC annealing)

Primer GCATGACAAGCTGGGC**C** produced the expected discrete band of 550 bp in lines of *S. eriocephala* and the line Juliet (thought to be *S. eriocephala* from ISSR and RAPD dendrograms) (Figure 4.14).

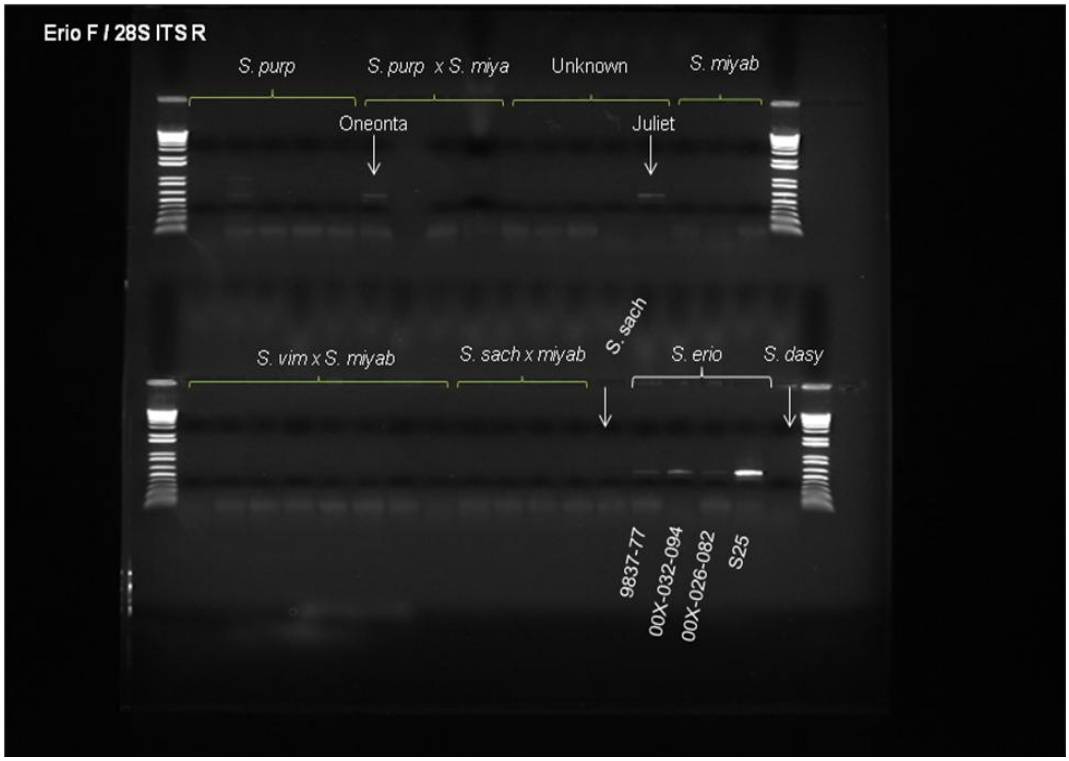


Figure 4.14: SCAR marker for *S. eriocephala*, Juliet and Oneonta (70ºC annealing)

The effect of progressive increase of annealing temperature was observed. As the annealing temperature increased, the amplified SCAR bands became specific to the targeted species (Figures 4.15-4.21). The 432bp band specific to *S. purpurea,* hybrid lines *S. purpurea x S. miyabeana* and the line Hotel became less intense as the optimal annealing temperature (64ºC) was reached (Figures 4.15-4.17).

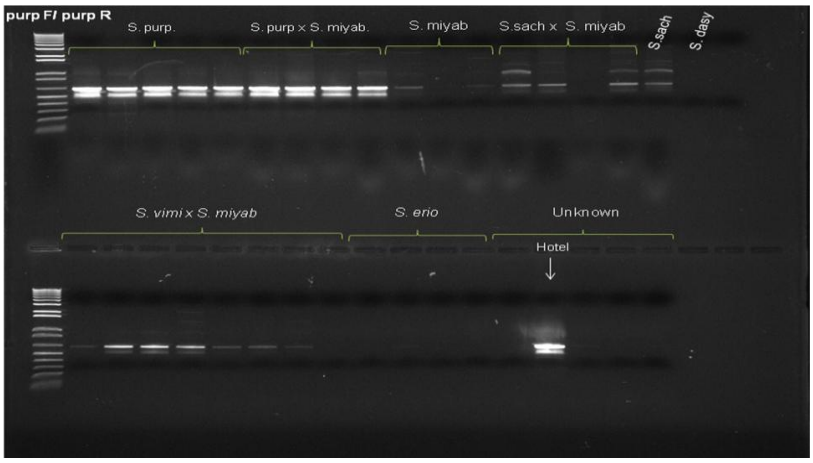


Figure 4.15: *S. purpurea* SCAR marker at 62ºC

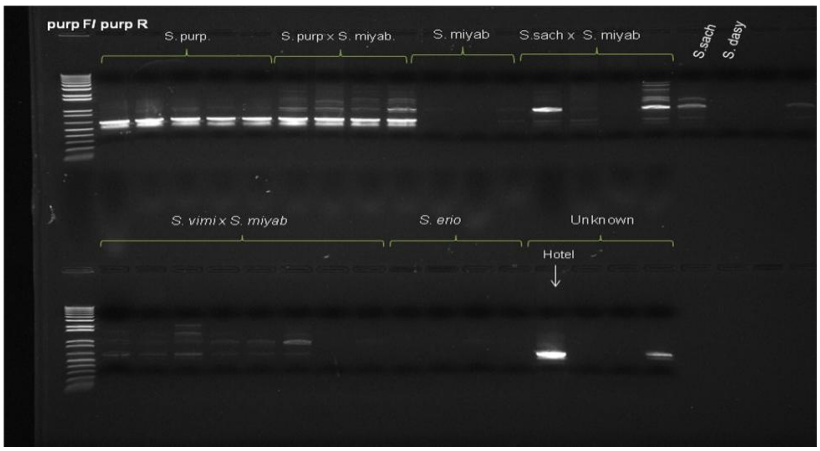


Figure 4.16: *S. purpurea* SCAR marker at 63ºC

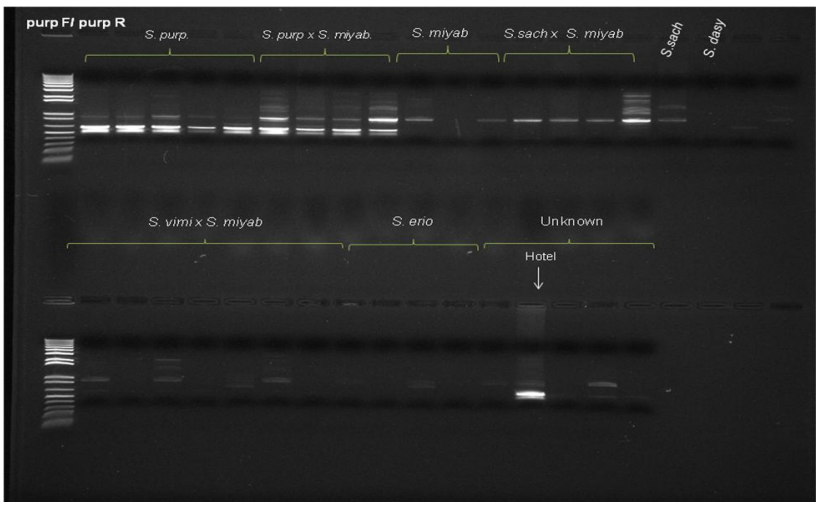


Figure 4.17: *S. purpurea* SCAR marker at 64ºC

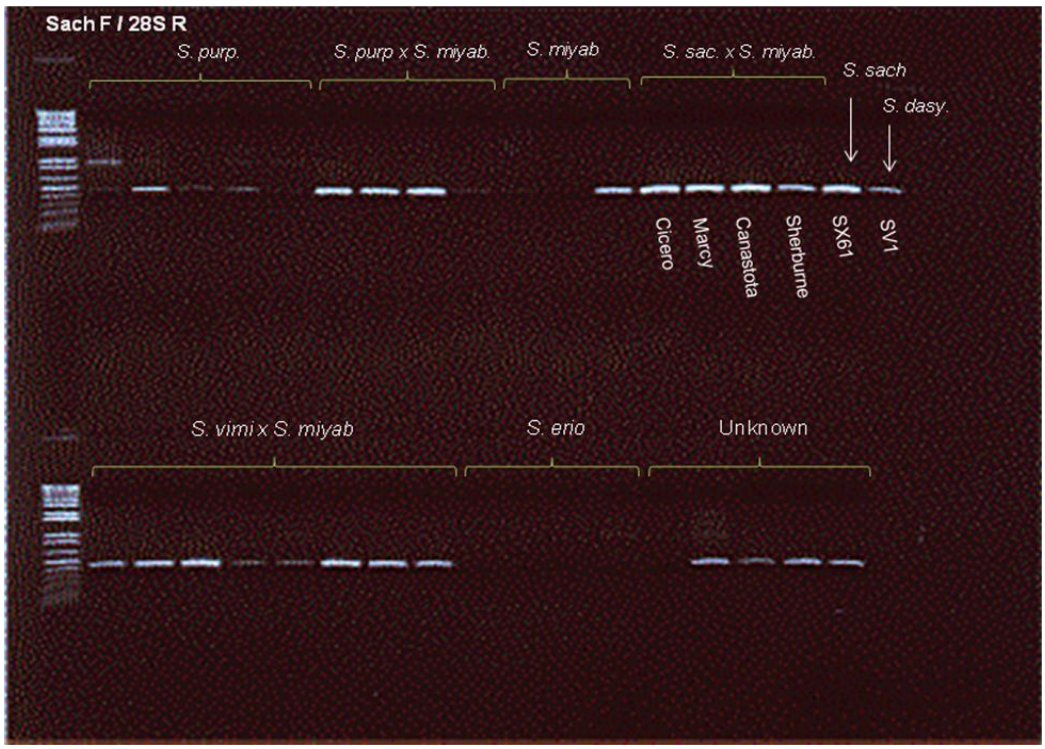


Figure 4.18 *S. sachalinensis* SCAR marker (67ºC annealing)

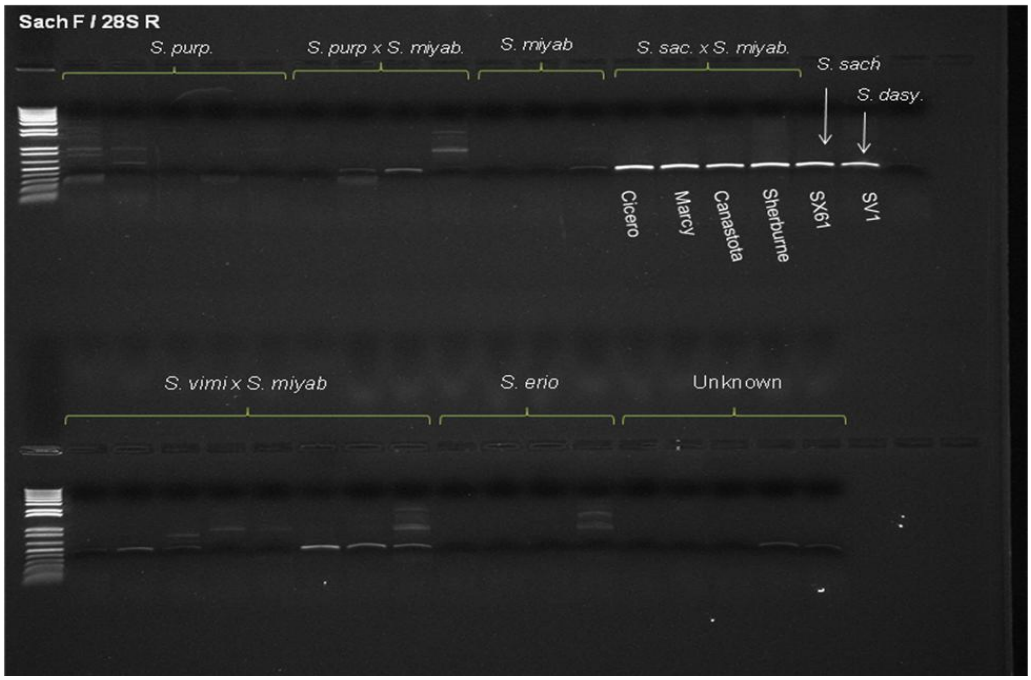


Figure 4.19 *S. sachalinensis* SCAR marker (68ºC annealing)

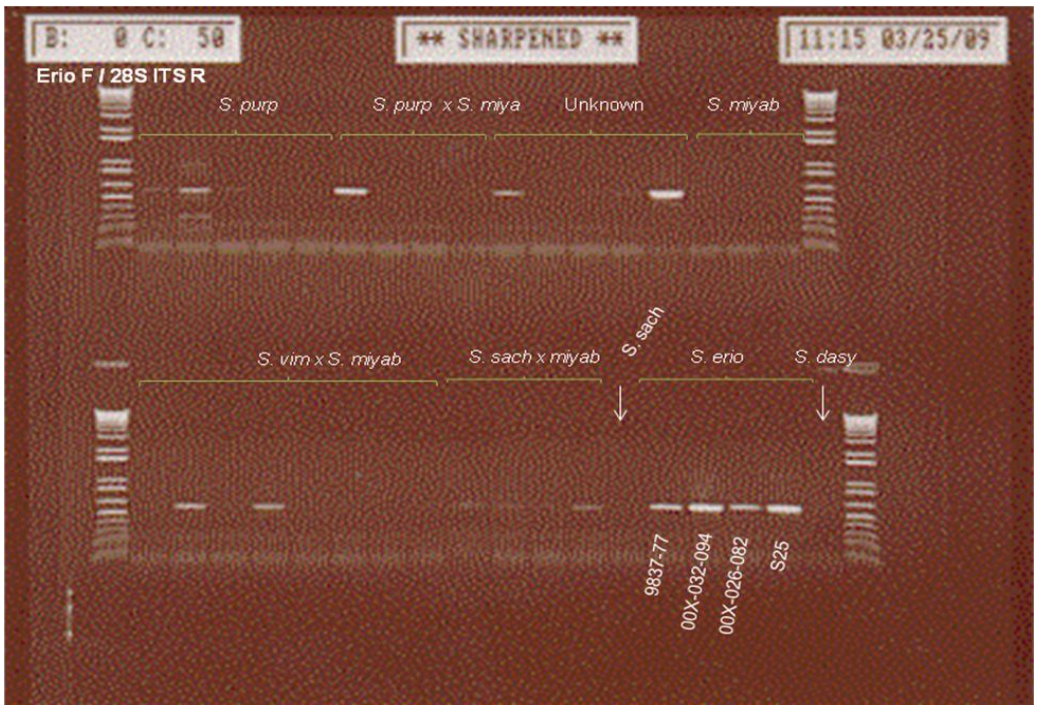


Figure 4.20 *S. eriocephala* SCAR marker (67ºC annealing)

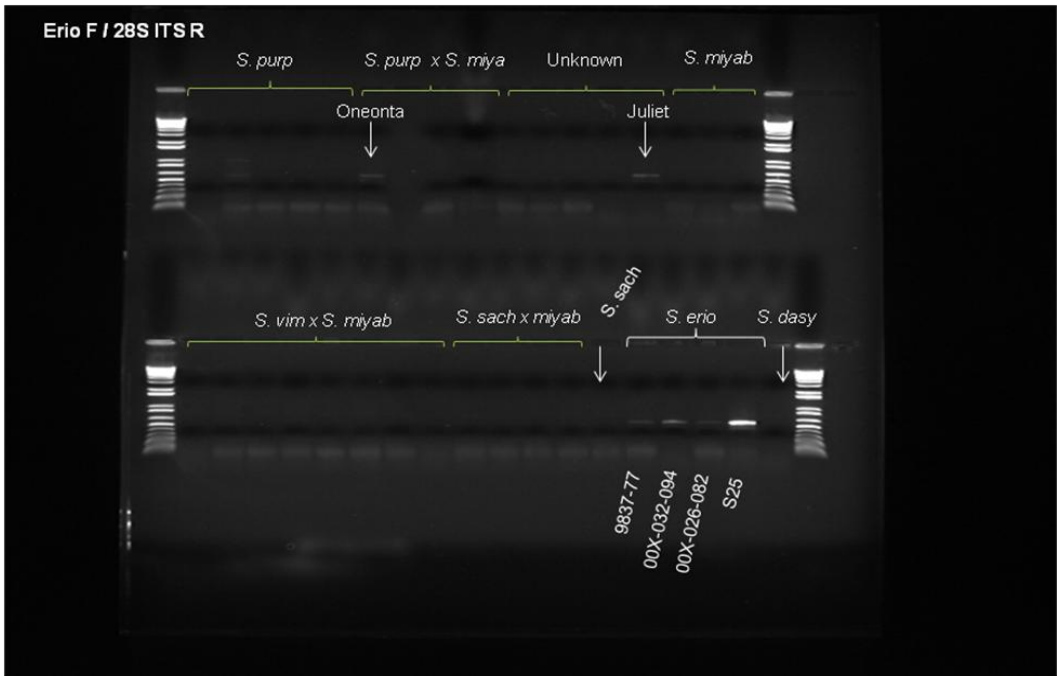


Figure 4.21 *S. eriocephala* SCAR marker (70ºC annealing)

4-5 Discussion and conclusions

In this study, the DNA region including ITS1, 5.8S, and ITS2 of 5 biomass willow lines belonging to 5 different *Salix* species (*Salix purpurea*, *Salix miyabeana*, *Salix eriocephala*, *Salix dasyclados*, *Salix sachalinensis*) was investigated. Nine polymorphic nucleotide sites were found, 6 within ITS1, and 3 within ITS2. The 5.8S gene did not show any polymorphism (Table 4.5).

Table 4.5 Comparison between sequenced ITS regions and the GenBank ITS sequence references (bolded bases represent nucleotide variation sites).

	SNP positions								
	ITS1						**ITS2**		
	7	**56**	**173**	**180**	**181**	**211**	**484**	**489**	**540**
Consensus ITS	A	T	A	G	T	T	A	C	T
S. purpurea	**G**	T	A	G	T	**C**	A	C	T
S.purpurea (NCBI)	A	T	A	G	T	**C**	A	C	T
S. eriocephala	A	**C**	A	G	T	T	A	C	T
S. eriocephala (NCBI)	A	**C**	A	G	T	T	A	C	T
S. dasyclados (S. dasy1)	A	T	**C**	G	T	T	A	C	T
S.dasyclados (NCBI)	A	T	A	G	T	T	A	C	**A**
S. dasyclados(S. dasy2)	A	T	**C**	G	T	T	**G**	**T**	T
S.viminalis (NCBI)	A	T	**C**	G	T	T	**G**	**T**	T
S. sachalinensis (S. sacha1)	A	T	A	**T**	**A**	T	A	C	**C**
S. sachalinensis (S. sacha 2)	A	T	A	G	T	T	A	C	**C**
S. miyabeana	A	T	A	G	T	T	A	C	**C**

The *S. purpurea* sequence found in this study differed from other ITS sequences with a G and a C at polymorphic sites 7 and 211 respectively, but differed as well from *S. purpurea* ITS sequence deposited in GenBank with a G instead of A at site 7 (Table 4.5). This could be due to polyploidy although only one *S. purpurea* ITS sequence was found in this study.

S. eriocephala sequence was the same as that in Genbank (Hardig and Brunsfeld, 2008), but differed from other ITS sequences with a C instead of T at site 56 (Table 4.5).

Three polymorphic nucleotide sites were found within ITS clones of *S. dasyclados,* leading to the identification of 2 ITS fragments. The first fragment (S. dasy1) had a C, a A and a C respectively on sites 173, 484 and 489, whereas the second (S. dasy2) had a C, a G and a T respectively. The second *S. dasyclados* fragment was the same as *S. viminalis* ITS sequence in Genbank (Leskinen and Alstron, 1999) (Table 4.5). This was an indication that either *S. dasyclados* or *S. viminalis* could be closely related, or a sign of a possible introgression of *S. viminalis* into the *S. dasyclados* genome. The first *S. dasyclados* ITS fragment (S. dasy1) was closer to the GenBank *S. dasyclados* ITS sequence (Leskinen and Alstron, 1999) (Table 4.5), but differed at site 173 and 540 where a C was found instead of an A, and a T instead of an A respectively (Table 4.5).

Two polymorphic nucleotide sites were found within ITS clones of *S. sachalinensis,* leading to the identification of two ITS fragments differing at sites 180 and 181. The first fragment had T and A, and the second fragment had G and T respectively (Table 4.5). Fragment 1 (S. sacha1) was unique to *S. sachalinensis* and was not found in any other published ITS sequence of *Salix* and *Populus* species (Leskinen and Alstron, 1999). The second fragment (S. sacha2) was much closer to the ITS sequences of *S. miyabeana* (Table 4.5). The presence of two rRNA loci could be an indication of the polyploidy of the investigated *S. sachalinensis* line. No *S. sachalinensis* ITS sequence has been published in GenBank.

The ITS sequence of *S. miyabeana* was identical to the overall consensus ITS sequence of the investigated *Salix* species (Table 4.5). No ITS sequence of *S. miyabeana* has been published.

The above mentioned nucleotide polymorphic sites could be used in combination to develop a species identification key for the investigated willow species (Figure. 4.22).

1-SNP 540

if T then go to 2

if C go to 5

2-SNPs 173, 484 and 489

if C, G and T, then ***S. dasyclados***

if A, A and C, then go to 3

3-SNPs 7 and 211

if G and C, then ***S. purpurea***

if A and T, then go to 4

4- SNP 56

if C then ***S. eriocephala***

5-SNPs 180 and 181

if T and A, then, ***S. sachalinensis***

if G and T, then ***S. miyabeana***

Figure 4.22 Species identification key based on combination of ITS1 and ITS2 polymorphic nucleotides

5- **GENERAL DISCUSSION AND CONCLUSION**

The dendrograms developed from RAPD and ISSR markers grouped different species and hybrids in clusters. The differences of banding patterns were used for the identification of willow lines. This confirms the efficiency of ISSR and RAPD marker techniques for genetic diversity analysis and for identification within *Salicaceae* reported by many authors (Rajora and Rahman, 2003; Gao et al., 2006; Sulima et al., 2009).

The determination of the total nuclear DNA content of different lines helped to confirm the groupings of species and hybrids observed on the dendrograms, and confirmed as well the correlations between nuclear DNA contents of hybrid and parental lines. The observed variability in ploidy levels within and between species have been reported in previous investigations on ploidy levels within the genus *Salix* (Suda and Argus, 1968; Thibault, 1998).

The analysis of rRNA gene regions including ITS1, 5.8S gene and ITS2 were useful for the identification of intra- and inter-specific nucleotide polymorphisms. Inter- and intra-species heterogeneity has been reported in many plants species (Saini et al., 2008; Hillis, 1988). The amplified ITS sequences were very similar to *Salix* ITS sequences published in GenBank (Leskinen and Alstron, 1999; Hardig and Brunsfeld, 2008).

The developed SCAR markers contributed to confirming the observed species-specific SNPs, as well as the species names of some lines of unknown origins. They appeared to be quicker to perform, easier to use and less expensive compared to SNP markers which necessitated PCR amplification, sequencing and sequence alignments.

The SCAR marker developed for *S. purpurea* had two bands of 624 and 432bp. The expected band (based on review of the DNA sequence) was the 624bp band. The 432bp band could be produced as a result of the fact that there is a potential annealing site (GGCGGA) for the reverse primer (**CCGCCT**GACCTGGGGT) located within the expected 624bp fragment, 172bp from the annealing site as shown on Figure 5.1.

Figure 5.1 Illustration of annealing sites of the reverse primer **CCGCCT**GACCTGGGGT which led to the production of two band sizes of 624bp and 432bp respectively, specific to *S. purpurea* and hybrid lines *S. purpurea x S. miyabeana*.

A number of lines were used in this study whose species designation was unknown. Both ISSR and RAPD dendrograms clustered the line Hotel with lines of *S. purpurea*. The SCAR bands of 624 and 432bp specific to *S. purpurea* and hybrids of *S. purpurea x S. miyabeana* were also present in line Hotel. The nuclear DNA content of Hotel fell within the range of nuclear DNA content of lines of *S. purpurea*. Thus Hotel is likely to be a line of *S. purpurea* or a hybrid line with *S. purpurea* as one parent and another species as the other parent. Similarly, both dendrograms grouped the line Juliet with lines of *S. eriocepala.* The SCAR band of 550bp specific to *S. eriocephala* was amplified in the line Juliet, and the nuclear DNA content of Juliet fell within the range of nuclear DNA of *S. eriocephala* lines. Thus, Juliet is likely to be a line of *S. eriocephala*. India was consistently grouped on both dendrograms with the only line of *S. dasyclados*, although their ploidy levels differed widely. Thus, India could be a polyploid line of *S. dasyclados*. The line S365 was also clustered with the line India and the only *S. dasyclados* in both dendrograms, but in both cases, line S365 had the lowest similarity index in the clusters. Thus, line S365 could be either *S. dasyclados* or a very close species. The line Charly had the lowest similarity index, approximately 50% in both dendrograms, and did not group with any cluster. Thus, the line Charly could be a distinct species not included in this study.

Strong and reproducible ISSR and RAPD bands were used in combination to identify individual lines of *S. purpurea*, hybrid lines *S. purpurea x S. miyabeana*, and hybrid lines of *S. sachalinensis x S. miyabeana* and the only line of *S. sachalinenesis*. This confirms efficiency of both techniques for species and cultivar identification reported in many publications (Prevost and Wilkinson, 1999; Rao et al., 2007). Overall,

the evaluation of 2C nuclear DNA through flow cytometry, ITS analysis, ISSR / RAPD markers were effective in revealing genetic diversity and to fingerprint willow species and lines used in the program.

6-NEXT STEPS

a) Optimise the species specific SCAR markers and confirm the consistency by applying them to other willows different from those used in this program.

b) Line and species-specific SCAR markers could be developed through the isolation, cloning and sequencing of line/species-specific RAPD/ISSR. A collection of such isolated bands has been constituted. Similarly, a collection of primers for the amplification of monomorphic ISSR/RAPD bands has been prepared (Table 6.1). Sequence alignment of such bands could reveal more SNPs.

Table 6.1 List of prepared primers for amplification of monomorphic RAPD/ISSR bands

Forward primer	**Reverse primer**
GTAATGAAAGATGACGT	CTGCTTGATGCCAAC
CGTAGTTCGTCACAAC	GCAGTAGAAAGTAATG
GTTGACAAGGGCTTC	CGAATTAATCTACCG
CCGAGCATCATGGCC	CTTCATTCCAATACTCGGTA
GAAGAACATACCCGGGTCA	CACGGGTCTTGTGGTAGG
GGGATAGACAGAAA	CGCTAATTAATTACAC
GTAATGAAAGATGACGTGTCATT	TCAACACTGCTTGATGCCAAC
GAGAGAGCTGACGAA	GTTACTCACATCTTATGG
CATAAGGAACAGCGATTCG	CTGAGGTTCAGAGGGTG
GGATATCAGCATGCG	GAAAGGTCTCTAGAAG
GGATATCAGCATGCG	AGCACCAAGTAATAAAC
CACCAGCCGCCTTTTATC	GTTACAGAGCAGTCGTCC

AFLP is known to generate more bands than RAPD and ISSR, and could be tested on this material for genetic diversity to confirm the clusters obtained with ISSR RAPD analysis, and could generate more line- and species- specific bands.

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7- APPENDICES

APPENDIX 1

CTAB DNA extraction protocol

1. Harvest 1 to 5 grams of plant tissue (fresh weight). Cut the tissue into small pieces into a pre-chilled mortar, immediately add liquid N. Grind the frozen tissue into a fine powder.

2. Transfer the frozen powder into a pre-chilled 50 mL polyallomer centrifuge tube. Keep the tube on ice.

3. Add 1 volume (15 mL) of warm (65° C) 2 X CTAB extraction solution. Mix well so that all of the powder is suspended (no lumps).

4. Incubate the extraction in a 65° C waterbath for 10 minutes. Mix the tubes once or twice during the incubation.

5. Add one volume of 24:1 chloroform/isoamyl alcohol. Mix thoroughly by inversion until the phases are mixed.

6. Centrifuge the extraction tubes at 2000 – 2500 rpm for 10 minutes.

7. Transfer 10 mL of the supernatant (top aqueous phase) to a clean polyallomer centrifuge tube. Discard the remaining liquid in the chloroform/IA waste bottle.

8. Add $1/10^{th}$ volume of warm (65° C) 10% CTAB solution. Mix well.

9. Repeat steps 4, 5, and 6.

10. Transfer 9 to 10 mL of the supernatant (top aqueous phase) to a 50 mL (Falcon type with screw cap) tube.

11. Add 2 volumes of cold (-20°C) 95% ethanol. Cap the tube. Mix the tube gently by inversion until the DNA precipitates. NOTE: The DNA will precipitate out of solution at this step. As the DNA precipitates, it becomes sensitive to shearing. Therefore, invert the tubes gently and DO NOT VORTEX MIX.

12. Place the tubes on ice (or in the freezer) for 30 minutes or longer. The extractions may be stored overnight at this point in the procedure.

13. Centrifuge the tubes for 1 to 5 minutes at 2000 rpm. The DNA should pellet at the bottom of the tube.

14. Pour off the ethanol, making sure that the DNA pellet stays in the tube.

15. Add 5 to 10 mL of cold 70% ethanol. Place the tubes on ice (or in the freezer) for 30 minutes or longer. The extractions may be stored overnight at this point in the procedure.

16. Centrifuge the tubes for 5 to 10 minutes at 2000 rpm. The DNA should firmly stick to the bottom of the tube.

17. Pour off the ethanol, making sure that the DNA pellet stays in the tube. Invert the tubes at a 45° angle to allow the remaining ethanol to drain, and the DNA pellet to dry.

18. Re-hydrate the DNA by adding TE buffer or double distilled water.

SOLUTIONS:

2 X CTAB Solution

2 % CTAB (w/v)
100mM Tris-HCl pH 8.0
20 mM EDTA pH 8.0
1% PVP (MW 44,000)
2.8 M NaCl
(autoclave for storage)

10% CTAB Solution

10 % CTAB (w/v)
1.4 mM NaCl
(autoclave for storage)

24:1 Chloroform:Isoamyl Alcohol

Chloroform (96 mL)
Isoamyl Alcohol (4 mL)

Appendix 2

List of ISSR primer sequences and annealing temperatures

Primer sequences	Annealing temperatures ºC
AGAGAGAGAGAGAGAGT	**55**
AGAGAGAGAGAGAGAGC	**55**
AGAGAGAGAGAGAGAGG	**55**
GAGAGAGAGAGAGAGAT	**55**
GAGAGAGAGAGAGAGAC	**55**
GAGAGAGAGAGAGAGAA	**55**
CTCTCTCTCTCTCTCTT	**50**
CTCTCTCTCTCTCTCTA	**50**
CTCTCTCTCTCTCTCTG	**50**
CACACACACACACACAT	**55**
CACACACACACACACAA	**55**
CACACACACACACACAG	**52**
GTGTGTGTGTGTGTGTA	**55**
GTGTGTGTGTGTGTGTC	**55**
GTGTGTGTGTGTGTGTT	**55**
TCTCTCTCTCTCTCTCA	**55**
TCTCTCTCTCTCTCTCC	**55**
TCTCTCTCTCTCTCTCG	**55**
ACACACACACACACACT	**55**
ACACACACACACACACA	**55**
ACACACACACACACACG	**55**
TGTGTGTGTGTGTGTGA	**55**
TGTGTGTGTGTGTGTGC	**55**
TGTGTGTGTGTGTGTGT	**55**
AGAGAGAGAGAGAGAGYT	**52**
AGAGAGAGAGAGAGAGYC	**52**
AGAGAGAGAGAGAGAGYG	**52**
GAGAGAGAGAGAGAGAYT	**52**
GAGAGAGAGAGAGAGAYC	**52**
GAGAGAGAGAGAGAGAYA	**52**
CTCTCTCTCTCTCTCTRT	**52**
CTCTCTCTCTCTCTCTRA	**52**
CTCTCTCTCTCTCTCTRG	**52**
ACACACACACACACRT	**52**
CCACACACACACACACRA	**52**
CTCTCTCTCTCTCTCTCRC	**52**
CTCTCTCTCTCTCTCTCRG	**52**
CACACACACACACACACART	**52**
CACACACACACACACACARC	**52**
CACACACACACACACACARG	**52**

R=(A,G); Y=(C,T)

Appendix 3

List of 15-base RAPD primer sequences

Code	Primer sequences
CMG 1501	GCCGTGGACTGCAGA
CMG 1502	CCCGACACCAGGTGA
CMG 1503	TCAGGTTATCGCCCC
CMG 1504	ACGCCGGTGCAGTCT
CMG 1505	GGCTATTCAGCTGGC
CMG 1506	GGCGCAATTCATGGC
CMG 1507	GCGATGACACAGGAC
CMG 1508	ACGGGGTTTACCGCT
CMG 1509	CCGATCACACAGGGT
CMG 1510	CGCAACAGCGCAGAG
CMG 1511	GTGGCTTCTGCAGCA
CMG 1512	GCCTGCCTGCTGACG
CMG 1513	GGAACCCAAGAGGAG
CMG 1514	GTGTGCCGGTGTAGG
CMG 1515	GTCCTGGAGCTGCGG
CMG 1516	GTCCTGGAGCTGCGG
CMG 1517	CTCTGCGGGTGCGAG
CMG 1518	GGCTGAGGTGTGTCG
CMG 1519	TGGGACGCGTGCACT
CMG 1520	CACGAGCGCAGTCCG
CMG 1521	ACCTGAGAGGGCCAC
CMG 1522	CAGAGGGGCACCTGG
CMG 1523	TGAGATCCCACACCC
CMG 1524	ATAGCGGCGTGCCAG
CMG 1525	AGCCTGTCGTACGTG
CMG 1526	GGACCACCGTAAGCC
CMG 1527	N/A
CMG 1528	GCAGATGGCACGGAG
CMG 1529	GCTCTGGCGCACCGA
CMG 1530	CACCCGTAGCGTGAG
CMG 1531	CAGCGCAGACATACC
CMG 1532	TGTCCGATGCTCGGC
CMG 1533	CTCGCCCATCCAGCC
CMG 1534	GACACGGCCCGATAG
CMG 1535	ACGATGGACCCTGAG

List of 10-base Operon primer sequences

Codes	Primer sequences
Operon J-1	CCCGGCATAA
Operon J-2	CCCGTTGGGA
Operon J-3	TCTCCGCTTG
Operon J-4	CCGAACACGG
Operon J-5	CTCCATGGGG
Operon J-6	TCGTTCCGCA
Operon J-7	CCTCTCGACA
Operon J-8	CATACCGTGG
Operon J-9	TGAGCCTCAC
Operon J-10	AAGCCCGAGG
Operon J-11	ACTCCTGCGA
Operon J-12	GTCCCGTGGT
Operon J-13	CCACACTACC
Operon J-14	CACCCGGATG
Operon J-15	TGTAGCAGGG
Operon J-16	CTGCTTAGGG
Operon J-17	ACGCCAGTTC
Operon J-18	TGGTCGCAGA
Operon J-19	GGACACCACT
Operon J-20	AAGCGGCCTC
Operon F-1	ACGGATCCTG
Operon F-2	GAGGATCCCT
Operon F-3	CCTGATCACC
Operon F-4	GGTGATCAGG
Operon F-5	CCGAATTCCC
Operon F-6	GGGAATTCGG
Operon F-7	CCGATATCCC
Operon F-8	GGGATATCGG
Operon F-9	CCAAGCTTCC
Operon F-10	GGAAGCTTGG
Operon F-11	TTGGTACCCC
Operon F-12	ACGGTACCAG
Operon F-13	GGCTGCAGAA
Operon F-14	TGCTGCAGGT
Operon F-15	CCAGTACTCC
Operon F-16	GGAGTACTGG
Operon F-17	AACCCGGGAA
Operon F-18	TTCCCGGGTT
Operon F-19	CCTCTAGACC
Operon F-20	GGTCTAGAGG

Codes	Primer sequences
Operon E-1	CCCAAGGTCC
Operon E-2	GGTGCGGGAA
Operon E-3	CCAGATGCAC
Operon E-4	GTGACATGCC
Operon E-5	TCAGGGAGGT
Operon E-6	AAGACCCCTC
Operon E-7	AGATGCAGCC
Operon E-8	TCACCACGGT
Operon E-9	CTTCACCCGA
Operon E-10	CACCAGGTGA
Operon E-11	GAGTCTCAGG
Operon E-12	TTATCGCCCC
Operon E-13	CCCGATTCGG
Operon E-14	TGCGGCTGAG
Operon E-15	ACGCACAACC